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Identification of Specific Cellular Genes Up-Regulated Late in Adenovirus Type 12 Infection

Andreas Dorn,[1,2,3] Hongxing Zhao,[3] Frederik Granberg,[3] Marianna Hösel,[1,2]† Dennis Webb,[2]† Catharina Svensson,[4] Ulf Pettersson,[3] and Walter Doerfler[1,2]*

Institute for Clinical and Molecular Virology, Erlangen University, Erlangen,[1] and Institute of Genetics, University of Cologne, Cologne,[2] Germany, and Rudbeck Laboratory, Department of Genetics and Pathology[3] and Department of Medical Biochemistry and Microbiology, BMC,[4] Uppsala University, Uppsala, Sweden

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The infection of human cells by adenoviruses leads to a gradual reduction in the activity of host cell functions while viral gene expression progresses in a regulated way. We used the DNA microarray technique to determine the transcriptional activity profiles of cellular genes upon infection with adenovirus type 12 (Ad12). The microarray data were validated by quantitative real-time PCR for genes which showed significant alterations after Ad12 infection. At 12 h postinfection, there is a striking up-regulation between 10- and 30-fold in the expression of the G1P2, IFIT1, and IFIT2 cellular immune response genes compared to mock-infected cells. At later stages of infection, when the majority of regulated cellular genes has been turned down, a limited number of cellular genes exhibit increased activities by factors of 3 or less. These genes belong to the signal transduction or transcriptional regulator classes or are active in protein degradation, like ANPEP, an aminopeptidase. The SCD and CYP2S1 genes function in lipid metabolism. The eucaryotic translation initiation factor 4 is up-regulated, and one of the major histocompatibility complex genes is diminished in activity. For two of the genes, one up-regulated (CTSF gene) and one down-regulated (CYR61 gene), alterations in gene activity were confirmed at the protein level by Western blotting experiments. Increased genetic activity of cellular genes late in adenovirus infection has not been reported previously and demonstrates that Ad12 has a sustained control of host cell gene expression well into the late phase of infection.

Within a few weeks, the injection of adenovirus type 12 (Ad12) into newborn Syrian hamsters (*Mesocricetus auratus*) induces undifferentiated tumors (11, 26) with neuroectodermal and mesenchymal characteristics in 70 to 90% of animals (9). The mechanism of oncogenesis in the animals is not understood. We have postulated and provided evidence for the notion that the insertion of multiple copies of Ad12 DNA into the genomes of the tumor cells can lead to fundamental perturbations in the structure of the cellular genomes with ensuing alterations in cellular DNA methylation and transcription patterns (7, 9, 18, 21). Since the alterations in cellular gene expression during tumor development and progression are likely to prove to be complex, we initiated studies to investigate the immediate effects of Ad12 in productively infected human cells by using the DNA microarray technique.

It has long been known that adenovirus infections of human cells elicit the shutoff of cellular genes late in the infection cycle (for reviews, see references 10 and 25). With DNA microarray technology, it has now become possible to investigate and quantitate cellular and viral transcription patterns in adenovirus-infected cells with greater detail and precision. A comprehensive overview of altered cellular transcription patterns in human cytomegalovirus-infected human cells has been obtained by global monitoring with oligonucleotide arrays (33). Differential gene expression in cells early after infection with the nononcogenic adenovirus type 2 (Ad2) has also been analyzed (16, 32), and a number of cellular genes have been implicated to be relevant for virus-host interactions. Cellular gene expression in Ad12-transformed cells (6) and in Ad12-induced tumors has also been investigated. In tumors induced by Ad12 in newborn hamsters, the cellular transcription patterns reveal both similarities and marked differences between individual tumors (9). The cDNA microarray technique has identified cellular genes that are differentially transcribed in Ad5- and Ad12-transformed cells (29, 30).

Adenoviruses are among the most efficient DNA viruses when replicating in cell culture. In productive infections, each cell can generate up to 10^4 progeny virions. Little is known about the mechanisms which contribute to this overproduction of new virions. Obviously, the efficient interaction of cellular factors with the viral replication machinery must be of importance and has been documented for viral DNA replication (for a recent review, see reference 15). Cellular apoptosis must be delayed long enough to ensure protracted production and accumulation of virions in the nuclei of the infected cells. Clearly, more work will be required before it will be possible to provide a complete description of virus-host interactions at the transcriptional and posttranscriptional levels.

In the present report, we analyzed viral and cellular gene activities late after infection in human cells productively in-

* Corresponding author. Mailing address: Institut für Klinische und Molekulare Virologie, Universität Erlangen-Nürnberg, Schlossgarten 4, D-91054 Erlangen, Germany. Phone: 49-9131-852-6002. Fax: 49-9131-852-6493. E-mail: Walter.Doerfler@viro.med.uni-erlangen.de.

† Present address: Center for Molecular Medicine, University of Cologne, Cologne, Germany.

fected with Ad12 by using DNA microarray techniques. In comparison to Ad2 or Ad5, Ad12 infection proceeds at a slower pace, with viral DNA replication commencing at 12 to 14 h postinfection (p.i.) (23). We therefore chose to investigate cellular gene transcription at 12, 24, 32, and 48 h p.i. The data obtained by using nylon membrane cDNA macroarrays and glass slides with cDNA microarrays have been validated by quantitative real-time PCR (Q-RTPCR). For these experiments, cytoplasmic RNA, which is likely to be translated in the infected cell, was used. At 12 h p.i., the transcription of cellular genes involved in the defense and/or immune response against virus infection is increased. At later time points, Ad12 infection leads mainly to transcriptional silencing of most cellular genes, while the expression of a number of cellular genes is specifically enhanced as late as 32 and 48 h p.i. compared to uninfected HeLa cells. The significance of the up-regulated functions for viral replication can be viewed in light of the prolonged transcription of many of the early and late Ad12-specific genes.

MATERIALS AND METHODS

Cells and virus. HeLa cells (American Type Culture Collection) were grown at 37°C in Dulbecco modified Eagle medium containing 10% fetal bovine serum equilibrated with 5% CO_2. Ad12 was propagated, and titers were determined as described previously (3). Plaque assays were performed according to published methods (8, 17).

Infection and RNA extraction. Cellular transcription patterns during the productive infection of HeLa cells with Ad12 were analyzed on DNA microarrays. HeLa cells grown to a density of 1×10^7 cells per 75-cm^2 plastic surface were infected with 25 PFU of Ad12 per cell in three independent experiments. At 12, 24, 32, and 48 h p.i., cells were harvested, and cytoplasmic RNA was isolated by using the concert cytoplasmic RNA reagent (Invitrogen, Carlsbad, Calif.). In parallel, the same amount of mock-infected HeLa cells was treated identically. The quality and quantity of RNA preparations were assessed in a bioanalyzer (Agilent Technologies) by using an RNA 6000 Nano LabChip kit.

DNA array experiments. Two different approaches were used for DNA array experiments. Initially and for survey experiments, we used nylon membranes purchased from Incyte Genomics (Palo Alto, Calif.). The major part of the analyses was, however, done with glass slide cDNA microarrays produced by the DNA microarray core facility, Uppsala University, Uppsala, Sweden.

(i) Nylon membrane array hybridization. Nylon membranes were prehybridized for 3 h at 65°C with 20 ml of hybridization solution (DIG Easy Hybmix; Roche, Basel, Switzerland) under gentle agitation. Cytoplasmic RNA (5 μg) was reverse transcribed in the presence of ^{33}P-labeled dCTP at 42°C for 2 h. The radioactively labeled cDNA was purified by centrifugation through Sephadex G50 columns. The labeling efficiency was determined in a scintillation counter. The cDNA was subsequently added to 20 ml of hybridization solution and heated to 95°C for 10 min to denature the cDNA. After 3 min of quenching on ice, the prehybridization solution was discarded, the denatured ^{33}P-labeled probe was added to the membrane, and hybridization was allowed to proceed for 16 to 18 h at 65°C. Subsequently, the membranes were washed at 68°C with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1% sodium dodecyl sulfate (SDS) followed by 0.6× SSC–1% SDS for 20 min each. The membranes were finally exposed to a phosphoimager plate for 18 h. Data evaluation was done by Incyte Genomics.

(ii) cDNA microarray hybridization. Each glass slide contained 7,500 genes (PCR products) spotted in duplicate and in different locations. The exact list of genes printed on the slides can be retrieved from http://www.genpat.uu.se/Forskargrupper/wcn/UU/InstrAndProd_section.htm. cDNAs were labeled by two different protocols, the Micromax labeling procedure (Perkin-Elmer, Wellesley, Mass.) for the 24-, 32-, and 48-h RNA samples and the Cyscribe postlabeling method (Amersham Bioscience) for the 12-h RNA samples. Five or 10 μg of RNA was used for reverse transcription with the Micromax or Cyscribe labeling protocols, respectively. For the labeling and hybridization techniques, the manufacturers' instructions were followed.

(iii) Data collection, normalization, and analysis. The arrays were scanned in a GenePix 4000B scanner (Axon Instruments, Union City, Calif.) at a resolution of 10 μm. The data were quantified by using the GenePix Pro 4.0 (Axon Instruments) analysis software. The quantitated arrays were analyzed with the freeware software R (http://cran.r-project.org/). The data were print-tip normalized, and after subtraction of background, the data were also normalized across all 18 slides. To determine and evaluate regulated genes, the software significance analysis of microarrays (27) was used.

Q-RTPCR. To validate the results obtained by the microarray analyses, the Taqman real-time PCR technique was performed on the same sets of RNA samples which had been used in the cDNA microarray experiments. In all experiments, 1 μg of the pooled cytoplasmic RNAs from each time point after infection were prepared, and the Superscript First-Strand synthesis system for reverse transcription-PCR (Invitrogen) was used for the synthesis of cDNA. PCR primers and Taqman MGB probes for the following genes were products from Assays-on-demand (Applied Biosystems, Foster City, Calif.): the CD83 (NM_004233), IFIT1 (NM_001548), G1P2 (NM_005101), interleukin 6 (IL-6) (NM_000600), JUNB (NM_002229), ATF2 (NM_001880), CYR61 (NM_001554), FUBP1 (NM_003902), CTSF (NM_003793), ANPEP (NM_001150), MCC (NM_002387), c-MYC (NM_002467), and ACTB (NM_001101) genes. The Q-RTPCR was performed following standard protocols (32) on an ABI PRISM 7700 sequence detection system (Applied Biosystems). Results were calculated and normalized relative to the β-actin control by using the Microsoft Excel program.

Western blot analysis. Total protein was isolated by using the T-PER reagent (12) according to the manufacturer's protocol. Isolated protein (25 μg) was applied to a 13% separating polyacrylamide gel. After electrophoresis, the proteins were transferred onto a polyvinylidene difluoride membrane (Amersham, Piscataway, N.J.) by using a Bio-Rad blotting chamber. The membranes were treated overnight at 4°C with blocking solution (0.2% Tween–5% milk powder in phosphate-buffered saline without Mg^{2+} and Ca^{2+} [PBSd]). The membranes were briefly washed in PBSd–0.2% Tween. Samples were incubated for 1 h at room temperature with the first antibody, CTSF (sc-13987; Santa Cruz Biotechnology) or CYR61 (sc-13100; Santa Cruz Biotechnology), in PBSd–0.2% Tween. The membranes were washed for 30 min at room temperature with two changes of washing buffer. Incubation with the secondary antibody, directed against the primary antibodies, was for 1 h at room temperature again in PBSd–0.2% Tween. Membranes were washed again for 30 min. To detect signals on the membranes, an ECL kit (Amersham) was used. Photos were taken with a Fujifilm LAS-1000 CCD camera (Fujifilm, Tokyo, Japan). Band intensities were determined by using AIDA image analysis software.

Single-stranded DNA probes and viral DNA arrays. Because both complements of adenovirus DNA encode genes, we isolated single-stranded DNA (ssDNA) probes by a new protocol (31) to analyze unequivocally viral transcription patterns. In brief, Ad12-specific DNA fragments corresponding to 28 open reading frames of Ad12 were amplified by PCR and cloned into M13mp18 DNA. M13-Ad12-specific ssDNA was isolated by using the ss-M13-DNA purification kit (QIAGEN, Hilden, Germany). Ad12 DNA-carrying M13 DNA (20 ng) was spotted on filters for HeLa-Ad12 DNA microarray analyses. Five micrograms of total RNA from mock- or Ad12-infected cells was reverse transcribed in the presence of 2 μg of oligo$(dT)_{12-18}$ and 1 mM concentrations of each of the deoxynucleoside triphosphates. After denaturation, 5 μCi of gamma-^{32}P-labeled dCTP (3,000 μCi/mmol), 10 mM dithiothreitol, reaction buffer, and 300 U of superscript II reverse transcriptase (Invitrogen) were added and allowed to react at 42°C for 1.5 h. Probes were purified by chromatography on Sephadex G-50 columns (Roche) and hybridized to DNA array membranes at 42°C for 20 h in digoxigenin hybridization buffer (Roche). Membranes were washed three times for 20 min in 0.5× SSC–1% SDS at 65°C and exposed to X-ray films for 14 h.

RESULTS

Patterns of Ad12-specific gene transcription. Ad12-specific transcripts were detected by DNA macroarray analyses using nylon membranes carrying single-stranded Ad12 DNA sequences from regions of the viral genome, as indicated for the controls in Fig. 1A. In mock-infected HeLa cells (Fig. 1A), none of the Ad12-specific transcripts could be found. It was ascertained that all spots on the microarray carried Ad12 DNA sequences, since they all hybridized with ^{32}P-labeled Ad12 virion DNA (data not shown). At 14 (31) and 24 (Fig. 1B) h p.i., transcripts from all early genes (E1 to E4) were detectable. Particularly high expression was observed from E1B and for RNAs corresponding to the 12.1- and 14.7-kDa E3 proteins.

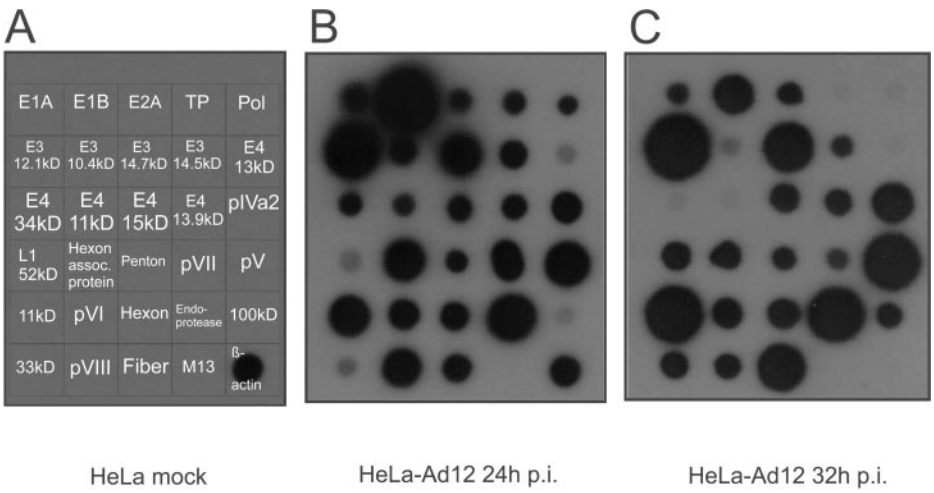

FIG. 1. DNA array analyses of Ad12 genes expressed in Ad12-infected HeLa cells. Cytoplasmic RNAs were isolated from mock-infected HeLa cells (A) or Ad12-infected HeLa cells at 24 h p.i. (B) or 32 h p.i. (C). The RNAs were reverse transcribed into cDNAs which were labeled with ^{32}P-labeled deoxynucleoside triphosphates. The grid in panel A indicates the positions of the single-stranded Ad12 DNA probes which were used to avoid possible ambiguities due to the complex coding profiles on the two complementary strands of adenovirus DNA. For experimental details, see Materials and Methods. TP, terminal protein; Pol, DNA polymerase.

Although some of the late gene transcripts were detected at 14 h p.i. (fiber, endoprotease, and major core), most were found only at 24 h p.i. However, transcription of the genes for the 52-kDa L1 and the 100- and 33-kDa L4 proteins was very low. At 32 h p.i. (Fig. 1C), RNA corresponding to the Ad12 terminal protein, DNA polymerase, and most of the E4 genes was no longer present. However, transcripts of the 12.1- and 14.7-kDa E3 genes and the pIVa2, minor core, 11-kDa, endoprotease, and fiber genes were abundant. The hexon and penton gene transcripts were almost equally transcribed at 24 and 32 h p.i. Obviously, at 32 h p.i. most of the assembly functions, pIVa2, L1, and the endoprotease as well as the fiber, hexon, penton, and 11-kDa proteins were strongly transcribed. Similarly, the transcripts of the genes for the precursor proteins pIVa2, pVI, and pVIII were abundantly represented at 24 and 32 h p.i., as well as the transcript of the gene for the endoprotease which was responsible for the maturation of the precursor proteins. The viral proteins IIIa, VI, VIII, and IX play a double role early during infection in that they stabilize and disassemble the capsid (22).

Even as late as 32 h p.i. (Fig. 1C), some of the genes for the E3 proteins, which were, at least in part, dispensable for the replication of adenoviruses in cell culture but antagonize cellular and immune defense functions, showed predominant transcription. Lastly, at 48 h p.i., the Ad12 transcription profile was not significantly different from that at 32 h p.i. (data not shown).

In Ad12-infected cells, the 14-h time point just marks the transition from the early to the late phase of the infection cycle. Significant changes in the late expression profile occur between 14 and 24 h p.i., with continued early gene expression up to 48 h p.i. These very late transcripts might be related to the observation of up-regulated cellular gene activity late in the infection cycle.

Overview of cellular gene transcription in Ad12-infected human HeLa cells at 24, 32, and 48 h p.i. As detailed in Materials and Methods, we initially chose nylon membranes carrying 8,400 cellular genes in duplicate to obtain an overall survey of cellular transcription patterns following Ad12 infection. About 6,000 of these genes were known human genes. Late in the infection cycle, the results shown in Fig. 2 documented the gradual and almost general switchoff of cellular gene expression. At 32 h p.i. (Fig. 2C) and at 48 h p.i. (Fig. 2D), >90% of the cellular genes on the membrane showed down-regulated transcription or no transcription at all in comparison to mock-infected HeLa cells (Fig. 2A) or to Ad12-infected HeLa cells during the first 24 h p.i. (Fig. 2B). Surprisingly, at 32 h p.i., about 1.3% of the genes on the membrane exhibited enhanced transcription by a factor of at least 3. At 48 h p.i. (Fig. 2D), the transcription of about 2% of the genes was up-regulated. The products of some of these genes might be required for efficient viral replication or for safeguarding against cellular apoptosis or had played a role in countering cellular mechanisms active in curtailing viral replication. Moreover, expression of some of the genes showing increased transcription at 32 h p.i. continued to increase even at 48 h p.i. In light of the sustained transcription of Ad12 genes at very late time points, we conclude that the Ad12-infected cell retains active transcription regulatory mechanisms for both viral and cellular genes as late as 48 h p.i. As an example of an up-regulated gene (the eucaryotic translation initiation factor 4 gene) and a down-regulated gene (the major histocompatibility complex [MHC] gene), an enlargement of membrane sections in Fig. 2A and C is shown (Fig. 3). E1A-mediated down-regulation of the class I MHC genes was demonstrated earlier in Ad12-transformed cells (1, 2, 5, 24, 28).

As controls, HeLa cells at 24 h after mock infection were used for comparisons to all time points of viral infection. This time point after mock infection was chosen as a compromise, since cells stop growing approximately 24 h p.i. Hence, cells overgrown at later times after mock infection could not have served as appropriate controls.

Quantitative analyses of alterations in cellular gene transcription in HeLa cells at 12, 24, 32, and 48 h after infection

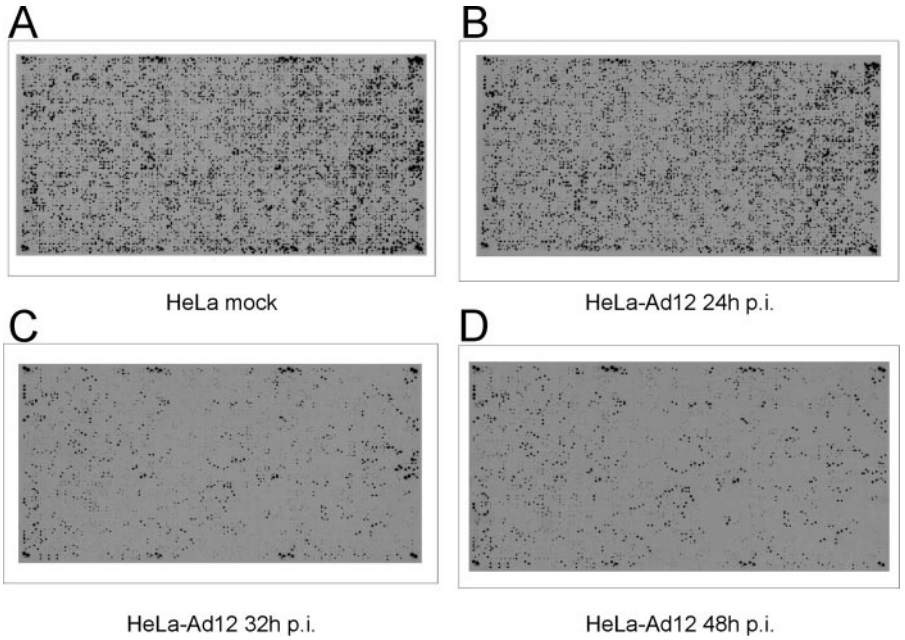

FIG. 2. Overview of the time course of human cellular gene transcription in mock-infected or in Ad12-infected HeLa cells at various times after infection. Cellular transcription patterns were determined by using membrane arrays containing 8,600 cellular genes. RNAs from the following sources were reverse transcribed into cDNAs and labeled with ^{33}P: mock-infected HeLa cells (A) and Ad12-infected HeLa cells at 24 h p.i. (B), 32 h p.i. (C), and 48 h p.i. (D). These cDNA preparations were then hybridized to the DNA arrays on the membranes. The decreasing number of expressed cellular genes and lower expression levels become apparent during the course of the infection.

with Ad12. Although the use of nylon membrane DNA macroarrays allowed for a qualitative visual overview of cellular transcription patterns after the infection of HeLa cells with Ad12, it proved difficult to quantify these data reliably. For the more detailed identification and quantitative analysis of specific changes in cellular gene transcription, we therefore turned to glass slide cDNA microarray analyses. cDNA preparations from reverse transcribed RNA from mock-infected or Ad12-infected cells, which were labeled with two different chromophores, were simultaneously hybridized to microarrays containing 7,500 cDNA clones. Three different sets of RNA samples from three independent time course experiments of mock- and Ad12-infected cells were prepared. Each RNA preparation was analyzed on two arrays in which the dye labeling was reversed (dye swap). Hence, the data for each time point were derived from six different microarrays. In the data presented here, the two complementary hybridization experiments yielded congruent results.

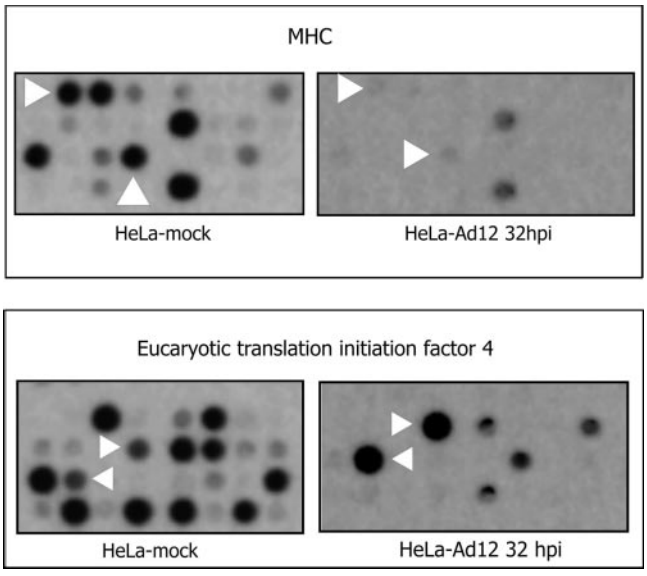

FIG. 3. Enlarged view of two selected segments from the arrays shown in Fig. 2A (mock infection) and C (32 h p.i.). Two examples for differentially regulated genes were presented: up-regulation of the eucaryotic translation initiation factor 4 and down-regulation of the MHC gene at 32 h p.i. Arrowheads indicate the exact positions of the genes on the nylon membranes.

The glass slide DNA microarray data were evaluated by using the software significance analysis of microarrays (27). Table 1 presents a number of cellular genes with significantly altered transcription in HeLa cells at 12, 24, 32, or 48 h after infection with Ad12 in comparison to mock-infected HeLa cells. In the first comparison, we analyzed transcription profiles at a time when the late phase of infection was clearly established (Fig. 2). This significance analysis included all genes which yielded signals in at least four of the six arrays at each time point. After the analysis, only genes with a false discovery rate lower than 4% were included in the final gene list. At 24 h p.i., 15 genes were negatively regulated by a factor of at least 2 relative to their transcription in uninfected HeLa cells. The same genes, as well as a few additional ones, were also down-regulated by the same criteria at 32 and 48 h p.i., many of them by factors similar to or even higher than those at 24 h p.i. (Table 1). The down-regulated genes included those for the transcription factors JUNB, NFKBA1, and NR4A1, the cell

TABLE 1. Changes in transcriptional activities upon the infection of HeLa cells with Ad12 as determined by the DNA microarray technique and in comparison to mock-infected HeLa cells[a]

Category	Clone ID	Gene	Gene name	Change (*n*-fold) compared to mock-infected HeLa cells at:			
				12 h p.i.	24 h p.i.	32 h p.i.	48 h p.i.
Immune response	742132	**GIP2**[c]	Interferon alpha-inducible protein (clone IFI-15K)	**24.3**	**1.0**[d]	**1.5**	**1.2**[d]
	289496	**IFIT2**	Interferon-induced protein with tetratricopeptide repeats 2	**9.4**	**−1.1**	**1.3**	**1.2**
	823696	**IFIT1**	Interferon-induced protein with tetratricopeptide repeats 1	**8.5**	**NSD**[b]	**NSD**	**NSD**
	530185	CD83	CD83 antigen	−1.7	−3.2	−1.2	−1.6
	627114	KLRC2	Killer cell lectin-like receptor subfamily C, member 2	NSD	−3.3	−1.8	−1.8
	310406	**IL-6**	Interleukin 6	**−1.7**	**−3.8**	**NSD**	**−3.7**
Signal transduction	51825	**RIS**	Ras family member Ris	**NSD**	**1.1**[d]	**2.4**	**1.7**
	1257170	PDE2A	Phosphodiesterase 2A; cGMP-stimulated	1.3[d]	1.5	1.8	1.3[d]
	241530	EPHA2	EphA2	1.0[d]	−2.6	−3	−2.3
	626343	SH3BP5	SH3-domain binding protein 5 (BTK-associated)	−1.6	−1.3	NSD	1.4
	1606557	FHL2	Four and a half LIM domains 2	−1.9	−1.1[d]	−1.5[d]	−1.2[d]
	293715	RAB1A	Member of RAS oncogene family	1.0[d]	−1.2[d]	−1.6	−1.2
	789376	TXNRD1	Thioredoxin reductase 1	−1.7	−1.2[d]	−1.2[d]	−1.4
	1358393	MAP2K3	Mitogen-activated protein kinase kinase 3	−1.3	−1.9	−2.6	−2.1
Transcription	293339	SNAI2	Snail homolog 2 (*Drosophila*)	1.6	−1.1[d]	NSD	−1.1
	949971	**ATF4**	Activating transcription factor 4	**1.0**[d]	**1.0**[d]	**1.8**	**1.9**
	26568	EGR3	Early growth response 3	1.0[d]	−1.3[d]	−1.8	−1.6
	299360	FUBP1	Far upstream element (FUSE) binding protein 1	1.0[d]	−1.1[d]	−1.5	−1.3[d]
	770910	ELF3	E74-like factor 3 (ets domain transcription factor)	−1.7	1.0[d]	1.1[d]	1.0[d]
	300482	FUBP3	Far upstream element (FUSE) binding protein 3	−1.2[d]	−2.0[d]	−1.6	1.0[d]
	309864	**JUNB**	jun B proto-oncogene	**1.4**	**−2.4**	**−2.3**	**−2**
	309893	**NR4A1**	Nuclear receptor subfamily 4, group A, member 1	**1.1**[d]	**−3.4**	**−3.5**	**−2.8**
	340734	**NFKB1A**	Nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor	**−1.1**∗	**−3.3**	**−2.1**	**−1.7**
	626716	ELL2	ELL-related RNA polymerase II, elongation factor	1.2[d]	−1.4[d]	−1.6	−1.3[d]
	682529	NFKB2	Nuclear factor of κ light polypeptide gene enhancer in B-cells 2	1.0[d]	−1.8	−1.1[d]	1.0[d]
	868575	**DSIPI**	Delta sleep-inducing peptide, immunoreactor	**1.1**[d]	**1.6**[d]	**2.3**	**2**
	884438	NFE2L2	Nuclear factor (erythroid-derived 2)-like 2	−1.4	−1.4	−1.5[d]	−1.5
	898312	TRAF4	Tumor necrosis factor receptor-associated factor 4	−1.2	−1.8	−1.3[d]	−1.1[d]
Cell cycle	2422905	MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	−2	1.4	1.3[d]	1.4
	199371	**CCND1**	Cyclin D1	**−1.6**[d]	**−1.3**[d]	**−3.4**	**−2.3**
	855949	CCNK	Cyclin K	−1.6	NSD	NSD	−1.7
	279670	BCAR3	Breast cancer anti-estrogen resistance 3	1.4	−2.2	−1.7[d]	−1.7
	795877	SNK	Serum-inducible kinase	1.1[d]	−2.9	−2.8	−2.8
Apoptosis	840776	SGK	Serum/glucocorticoid-regulated kinase	−1.8	−1.4[d]	−1.3[d]	−1.1[d]
	3033304	MCL1	Myeloid cell leukemia sequence 1 (BCL2-related)	−1.1	−1.7	−2	−1.9
	810724	IER3	Immediate early response 3	−2	−3.3	−2.5	−2.4
	1759582	TNFRSF12A	Tumor necrosis factor receptor superfamily, member 12A	−1.2[d]	−1.5	−2.4	−3.5
Chromatin structure	290841	HIST1H2BK	Histone 1, H2bk	1.8	−1.1[d]	1.2[d]	1.5[d]
	343744	HIF0	HI histone family, member 0	−1.4	−2.2	NSD	NSD
	1836558	HIST1H2AE	Histone 1, H2ae	−1.5	NSD	NSD	NSD
	701089	HIST1H4B	Histone 1, H4b	−2	NSD	NSD	NSD
Cell structure	291880	MFAP2	Microfibrillar-associated protein 2	NSD	−1.6	−1.1[d]	−2.5
	378488	**CYR61**	Cysteine-rich, angiogenic inducer, 61	**NSD**	**−3.8**	**−6.1**	**−7**
	814546	PSCD1	Pleckstrin homology, Sec7 and coiled-coil domains 1	NSD	−2.1	−2.1	−1.8[d]
	897768	COL7A1	Collagen, type VII, alpha 1	−1.3[d]	−1.9	−1.8	−1.4[d]
	Rnd3	Rnd3	Rnd3	1.1[d]	−2.3	−2.6	−2.2
	183602	**KRT14**	Keratin 14	**2.2**	**1.0**[d]	**−1.4**	**−1.9**
	810131	**KRT19**	Keratin 19	**2.1**	**−1.1**[d]	**−1.5**	**−2**
	841415	EPLIN	Epithelial protein lost in neoplasm beta	−1.7	−2	NSD	NSD
	2180765	**SDCBP**	Syndecan binding protein (syntenin)	**−1.1**[d]	**−2.6**	**−2.8**	**−1.9**
Cell metabolism	511633	**RIG-I**	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide	**5.1**	**NSD**	**NSD**	**NSD**
	135449	EWSR1	Ewing sarcoma breakpoint region 1	−1.2	−1.2[d]	−1.6	−1.1[d]
	80399	SFRS1	Splicing factor, arginine/serine-rich 1 (splicing factor 2)	1.0[d]	−1.2	−1.6	−1.2[d]
	358457	HNRPH1	Heterogeneous nuclear ribonucleoprotein H1 (H)	−1.3[d]	1.0[d]	−1.6	−1.3
	626531	NSAP1	NS1-associated protein 1	−1.2[d]	1.0[d]	−1.6	−1.3
	898265	SFRS5	Splicing factor, arginine/serine-rich 5	−1.3[d]	−2.5	−3.2	−2.4
	825470	TOP2A	Topoisomerase (DNA) II alpha, 170 kDa	−1.2[d]	−1.1[d]	1.6	1.3
	2499237	SERPINB5	Serine (or cysteine) proteinase inhibitor, clade B, member 5	NSD	1.0[d]	−1.1[d]	−1.6
	1474684	EFNA1	Ephrin-A1	−2.0[d]	−3.7	−2.9	−3.2
	82676	ANPEP	Aminopeptidase N	−1.1[d]	1.1	1.9	1.8
	784589	**MMP15**	Matrix metalloproteinase 15 (membrane-inserted)	**2.8**	**NSD**	**NSD**	**NSD**
	252515	KYNU	Kynureninase (L-kynurenine hydrolase)	−1.7	NSD	NSD	NSD
	810454	BACE2	Beta-site APP-cleaving enzyme 2	−2.2	1.1[d]	1.1[d]	1.2

Continued on facing page

TABLE 1—*Continued*

Category	Clone ID	Gene	Gene name	Change (*n*-fold) compared to mock-infected HeLa cells at:			
				12 h p.i.	24 h p.i.	32 h p.i.	48 h p.i.
	2496262	NPR3	Natriuretic peptide receptor C	1.4	1.0[d]	−1.1[d]	−1.5
	83605	CPS1	Carbamoyl-phosphate synthetase 1, mitochondrial	−2	−1.1[d]	1.2	1.5
	796646	ODC1	Ornithine decarboxylase 1	−1.1	−1.4	−1.6	−1.4
	525221	**ALDH3A1**	Aldehyde dehydrogenase 3 family, member A1	**NSD**	**1.0**[d]	**1.9**	**1.8**
	85259	HMOX1	Heme oxygenase (decycling) 1	−1.7	−2	−2.8	−2.1
	841689	ATP6IP1	ATPase, H^+ transporting, lysosomal interacting protein 1	1.0[d]	1.0[d]	1.6	1.4
	626967	ALP1	Alkaline phosphatase, intestinal	−2.3	NSD	NSD	NSD
	810711	**SCD**	Stearoyl-CoA desaturase (delta-9-desaturase)	**−1.7**[d]	**−1.1**[d]	**1.6**	**2.1**
	897733	SLC38A2	Solute carrier family 38, member 2	−1.6[d]	−1.5	−1.1[d]	−1.2
	877613	DCTN1	Dynactin 1 (p150, glued homolog, *Drosophila*)	1.0[d]	1.1[d]	1.8	1.3[d]
	1639732	PRKACG	Protein kinase, cyclic AMP-dependent, catalytic, gamma	1.0[d]	−1.9[d]	−1.7	−1.4[d]
	1574926	**CYP2S1**	Cytochrome P450, family 2, subfamily S, polypeptide 1	**NSD**	**1.4**[d]	**1.9**	**1.5**
	825295	LDLR	Low-density lipoprotein receptor	−1.4	−1.7[d]	−1.4	NSD
	2448778	**CTSF**	Cathepsin F	**1.1**[d]	**1.2**[d]	**2.6**	**2.2**

[a] Experimental details were described in the text.
[b] NSD, No signal detectable on the array.
[c] Boldfaced entries refer to genes discussed in detail in the text.
[d] Values with high variation. Bold values and genes indicate that the coefficient of variation was >90%.

cycle regulator CCND1, and several cell surface receptors and apoptosis and immune response regulators. The strongest down-regulation of the cell adhesion molecule CYR61 together with the down-regulation of a number of the cytoskeletal genes, such as the SDCBP gene, were consistent with the severely compromised cellular organization of cells late in Ad12 infection.

In addition to this finding, and functionally important, the transcription of several cellular genes was up-regulated at 32 and 48 h p.i. The transcription of the RIS, ATF4, DSIPI, ANPEP, SCD, ALDH3A1, CYP2S1, and CTSF genes (Table 1) was significantly enhanced at 32 and 48 h p.i. Importantly, these effects were essentially absent at 24 h p.i., and this finding supports the late up-regulation of specific cellular genes which was observed in the nylon filter cDNA macroarray experiments (Fig. 2). The identities of these cellular genes placed them among the signal transduction or transcriptional regulator classes (RIS, DSIPI, and ATF4) and genes for protein degradation (CTSF and ANPEP genes) and lipid metabolism (SCD and CYP2S1 genes).

In the second comparison, we analyzed cells at 12 h p.i., which corresponded to the time in the viral infection cycle when viral DNA replication commenced but when late viral gene expression had not yet begun. The cellular transcription profile at 12 h p.i. was found to be significantly different from that at the late phase of infection. Approximately 30% of the identified genes were found to be up-regulated. In particular, the expression of three cellular immune response genes, the G1P2, IFIT1, and IFIT2 genes, was increased between ~10- and 30-fold (Table 1). A strong induction was also observed for the RIG-I helicase gene and the MMP15 metalloproteinase gene. The KRT14 and KRT19 cytoskeleton genes were induced at 12 h p.i. but progressively repressed at later time points. Among the cellular genes that were down-regulated at 12 h p.i., repression rarely exceeded twofold. Adenovirus infection was previously shown to inhibit the transcription of histone genes (4).

Validation of the alterations in cellular gene transcription by Q-RTPCR. The significance of the results obtained by DNA microarray analyses was validated by Q-RTPCR. Generally, the same RNA preparation was used for both the Q-RTPCR and the cDNA microarray analyses. The transcriptional activity was normalized to the activity of the β-actin gene, whose expression was not noticeably affected by Ad12 infection. The results obtained for several cellular genes are summarized in Table 2, which also presents a quantitative comparison to the data obtained from the microarray system. For all genes analyzed, except for the late expression of the G1P2 gene, the results obtained by the two independent methods proved satisfactorily congruent. Notably, the relative change in expression of IL-6 was more pronounced by Q-RTPCR analysis than by the microarray data. The MCC gene was not present on the cDNA microarrays but was included in the results obtained from the nylon membrane experiments. Upon Ad12 infection of HeLa cells, the cellular MCC, CTSF, and ANPEP genes were markedly up-regulated, as documented by both the array and Q-RTPCR analyses (Tables 1 and 2). The majority of cellular genes was down-regulated after Ad12 infection, and this finding was corroborated by Q-RTPCR for the cellular JUNB, ATF2, IL-6, c-MYC, CYR61, and FUBP genes (Tables 1 and 2).

Western blot analysis. In order to investigate and confirm the changes in transcription activities, the protein levels of one up-regulated gene (the CTSF gene) and one down-regulated gene (the CYR61 gene) were analyzed by the Western blotting technique (Fig. 4). There were no major differences in the amount of CTSF protein between mock-infected cells and HeLa cells infected with Ad12 at 24 or 32 h p.i. At 48 h p.i., however, the CTSF protein levels increased by a factor of 5 compared to mock-infected cell protein levels. For CYR61, the opposite result was obtained (Fig. 4). Up to 48 h p.i., the CYR61 protein level remained constant and comparable to that in mock-infected cells. However, at 48 h p.i., the amount of CYR61 protein dropped by a factor of around 4 compared

TABLE 2. Cellular gene expression as analyzed by Q-RTPCR compared to microarray analyses

Gene	Change (*n*-fold)[a]							
	Q-RTPCR				Microarray[b]			
	12 h p.i.	24 h p.i.	32 h p.i.	48 h p.i.	12 h p.i.	24 h p.i.	32 h p.i.	48 h p.i.
β-Actin	1	1	1	1	1	1	1	1
G1P2	**44**	−6.5	−1.9	−2.0	**24.3**	**1.0**	**1.5**	**1.2**
IFIT1	**15**	**1.1**	**2.1**	**1.5**	**8.5**	NSD[e]	NSD	NSD
CD83	1.05	−2.2	−1.5	−1.1	−1.7	−3.2	−1.2	−1.6
JunB	n.a.[d]	−2.5	−2.1	−4.4	**1.4**	−2.4	−2.3	−2.0
ATF2	−1.1	−0.9	−1	−1.8	**1.0**	−1.2	NSD	−1.2
MCC[c]	1.2	**1.6**	**2.6**	**1.6**				
IL-6	1.7	**−41**	−14.9	−21.9	−1.7	−3.8	NSD	−3.7
c-Myc	−1.4	−2.5	−1.6	−2.8	−1.8			
CTSF	n.a.	**1.4**	**2.8**	**3.3**	**1.1**	**1.2**	**2.6**	**2.2**
ANPEP	n.a.	**0.8**	**1.4**	**1.5**	−1.1	**1.1**	**1.9**	**1.8**
CYR61	1.2	−2.0	−1.8	−2.4	NSD	−3.8	−6.1	−7.0
FUBP	n.a.	−1.0	−0.6	−0.8	1.0	1.0	−1.5	−1.3

[a] The factors in this table relate gene activities in Ad12-infected cells to those in mock-infected cells. All factors in this table were normalized relative to the activity of the β-actin gene, whose activity did not change in HeLa cells after Ad12 infection. Boldfaced entries indicate genes discussed in the text.
[b] These data were taken from Table 1.
[c] The MCC gene was not present on the DNA microarray.
[d] n.a., not analyzed.
[e] NSD, no signal detectable.

to mock-infected cells. For both genes, the amounts of protein corresponded well to the results obtained by microarray and Q-RTPCR analyses (Table 2). In conclusion, the increased transcriptional activity of the CTSF gene at 48 h p.i. also led to a marked overproduction of the gene product. A similar congruence between the decreased transcriptional and translational activities was also confirmed for the CYR61 gene. In further analyses of the Ad12 system, it will be important to address changes in translational levels in more detail.

DISCUSSION

Changes in transcriptional levels of cellular genes upon Ad12 infection: functional implications. Ad12 DNA replication in productively infected HeLa cells begins at about 12 to 14 h p.i. (23). At 12 h p.i., the transcription of the cellular defense genes against viral infection, like those for G1P2, IFIT1, and IFIT2, is markedly up-regulated (Tables 1 and 2). As a response to this immediate antiviral maneuver of the infected cell, the viral genome appears to activate the full transcription of its E3 genes already at 14 h p.i. High transcription levels of the E3 genes are maintained throughout the infection cycle (Fig. 1C). Other cellular genes, whose transcription levels are turned up at 12 h p.i., belong to the helicase, cytoskeleton, and peptidase gene groups. In contrast, many cellular genes from the signal transduction, chromosome organization, cell cycle control, or apoptosis pathways are down-regulated at 12 h after infection with Ad12 and down-regulated more extensively at later times.

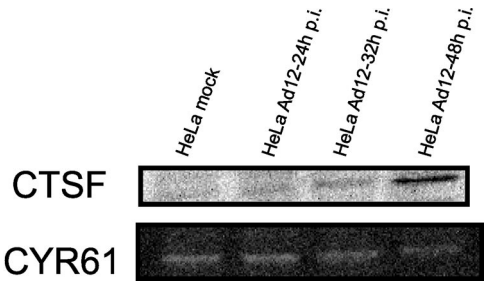

FIG. 4. Western blot analyses for two of the differentially expressed cellular genes. As described in Materials and Methods, total cellular protein was prepared from HeLa cells at 24, 32, and 48 h p.i. with Ad12. Mock-infected HeLa cells were also analyzed. By using specific antisera, specific protein expression was assessed by electrophoresis on polyacrylamide gels and by Western blotting. Increased production of CTSF and decreased production of CYR61 could be documented at 48 h p.i. These results are in good agreement with the data obtained by Q-RTPCR analyses on the transcription of these cellular genes (Table 2).

One of the strongly down-regulated genes throughout the productive infection of human HeLa cells with Ad12 was the CYR61 gene, also known as CCN1. This gene belongs to the family of CCN proteins (CYR61/CTGF/NOV) and encodes a 379-amino-acid polypeptide which is associated with the extracellular matrix and the cell membrane. One of its functions includes interaction with multiple integrins in order to maintain cell adhesion (14, 20), as shown by the use of antibodies against CYR61, which effectively inhibits cell adhesion. As shown in Table 1, additional cell adhesion genes were also down-regulated. By down-regulating these genes, the virus might effect a decrease in cell adhesion and the loosening of tissue organization. As a consequence, the adenovirus-specific cytopathic effect can develop more efficiently and spread throughout the infected cell culture or tissue. Thus, the virus is released over a broader area of the cell monolayer or the infected tissue and will be able to infect neighboring cells. Another member of the CCN family, the connective tissue growth factor (CTGF), has recently been shown to be up-regulated in Ad2 E1A-positive human bronchial epithelial cells (19). It remains to be determined whether this finding is relevant for the difference in oncogenicity between Ad2 and Ad12.

Among the cellular genes with up-regulated transcription late in the infection cycle, ANPEP and CTSF have functions in

protein degradation which, if directed against cellular proteins, could facilitate the release of assembled virions from the infected cell. It may be relevant that Ad2 infection also induces the expression of two lysosomal proteases, CTSD and CTNS (32).

Moreover, several transcriptional regulator genes are targeted by the virus and are down-regulated during the late phase of Ad12 infection. At all late time points analyzed, these genes show a significantly lower transcription level than in mock-infected cells. However, at 12 h p.i., neither JUNB nor NFKB1A/NFKB2 is down-regulated. During the early phase of infection of HeLa cells with Ad2, these transcription factors are up-regulated (32). This up-regulation might help drive the Ad2-infected cell into the S phase of the cell cycle and thus contribute to the more rapid infection cycle of Ad2 compared to Ad12. Significantly, at later times in Ad2 infection, NFKB1A and NFKB2 are both repressed (H. Zhao, F. Granberg, C. Svensson, and U. Pettersson, unpublished data). Hence, it is conceivable that, after the infected cells have entered S phase, at least some of the transcriptional regulators are repressed, so they would not interfere with the ongoing virus infection and the transcription of viral genes. Since these genes are involved in many different transcriptional pathways, it might be a necessity for the virus to repress them in order to implement its own transcriptional programs and effective replication cycle.

In a parallel project, we investigated cellular transcription profiles following Ad2 infection. A comparison of cellular transcription activities between Ad12 and Ad2 demonstrated a number of genes or gene classes that were similarly regulated. In particular, cellular genes involved in cell cycle control (the CCND1 and SGK genes) and stress response (the SNK and IL-6 genes) were repressed by both Ad2 and Ad12 infections. Moreover, the antiapoptotic SNAI1/SNAI2 was induced by infection with either virus (reference 32 and this analysis).

Transcription of the MCC gene was up-regulated 1.6- to 2.6-fold (Table 2). The MCC gene is a candidate for the putative colorectal tumor suppressor gene located at 5q21 in the human genome (13). The MCC gene encodes an 829-amino-acid protein with a region of similarity to the G protein-coupled muscarinic acetylcholine receptor. According to the SOURCE database (available at http://source.stanford.edu/), the gene might be involved in signal transduction as well as in the negative regulation of the cell cycle.

This investigation into alterations in the transcription of cellular functions in the course of productive infection of human cells by Ad12 has been initiated to elucidate the complex programs which govern the interaction of adenoviruses with their human host cells. A number of changes in cellular transcription patterns have been reliably and reproducibly documented. We continue to investigate the delicate, finely tuned temporal interplay between the activities of cellular and viral genes at different times after Ad12 infection and in Ad12-induced tumors.

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ADDENDUM IN PROOF

We have now investigated the protein levels of an additional strongly up-regulated gene, G1P2, also known as ISG15, at various times after the infection of HeLa cells with Ad12. The data from DNA array analysis and Q-RTPCR indicated that, after strong up-regulation at 12 h p.i., transcription of the gene at later times was down-regulated (Table 2). In contrast, the synthesis of the G1P2 protein increases continuously throughout infection. At 12 h p.i., the amount of G1P2 protein is augmented 9.2-fold; at 24 h p.i., 8.9-fold; at 32 h p.i., 13.4-fold, and at 48 h p.i., 13.9-fold compared to that of mock-infected HeLa cells. Apparently, the translation of even small amounts of G1P2-specific mRNA continues robustly in the course of productive Ad12 infection.

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