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Interactions among regulators of RNA abundance characterized using RNA fingerprinting by arbitrarily primed PCR

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ABSTRACT

Using RNA fingerprinting by arbitrarily primed PCR it is possible to infer convergent transcript regulatory pathways from the coordinate behavior of subsets of anonymous transcripts without cloning any genes. The number of transcripts in each response category can be estimated. The same may be true for differential display. We demonstrate these claims by treating a cell line with two known modulators of RNA abundance, transforming growth factor-β (TGFβ) and cycloheximide (CX), used together and alone. The responses of over 1700 anonymous transcripts were monitored under these three conditions and in an untreated control. Eight of the twenty-seven [3^3] possible transcript response categories were observed among 86 differentially expressed transcripts. For example, CX stabilizes or induces as many as 2.7% of transcripts of which about one third do not accumulate when TGFβ is also present. This intersection may reflect CX stabilization or induction of an important class of RNAs that otherwise usually have short half-lives. We predict that RNAs in this class constitute the majority of transcripts targeted for rapid down regulation in response to TGFβ and perhaps most other natural transcriptional modulators.

INTRODUCTION

The majority of early events in signal transduction pathways involve protein–protein interactions, protein modification, and communication via second messengers, resulting, ultimately in modulation of RNA transcript abundances and protein abundances. In this report we present a strategy for estimating the number of genes in different transcript regulatory categories, identifying new regulatory categories and using this information to infer general characteristics of signal transduction pathways. Of particular interest are transcripts that are modulated by more than one effector. Such intersections in modulation indicate intersections in the corresponding regulatory pathways.

Arbitrarily primed PCR allows the generation of reproducible fingerprints from DNA (1,2) or RNA (3,4). RNA fingerprinting by arbitrarily primed PCR (RAP-PCR) or differential display can be used to compare the abundances of about 100 transcripts per lane on a gel from many RNA populations in parallel (3,4). This method allows many thousands of RNAs to be compared in a partly abundance normalized fashion (5,6). The method provides a complex phenotype reflecting changes in the abundances of anonymous RNAs under various conditions. Comparison of fingerprints from many different treatment groups, in parallel, allows one to draw inferences regarding gene regulation. Hypotheses regarding signal transduction can be tested and new hypotheses generated using this information. When new categories of regulation are discovered, fragments of differentially expressed genes in these categories can be cloned directly from PCR amplified products.

To illustrate this idea we choose the simplest case of two modulators, transforming growth factor beta (TGFβ) and cycloheximide (CX), which can be used to investigate twenty-seven possible regulatory categories (Table 1). TGFβ causes some cell types to arrest in G_1. CX inhibits protein synthesis and can be used to categorize genes that respond to TGFβ-treatment into two groups, those that do not and those that do require protein synthesis in order to respond (i.e., primary and secondary responses, respectively). In addition CX is known to stabilize some otherwise rapidly degraded RNAs (7,8) and induce transcription of other genes (9–12). We set out to measure the proportion of transcripts regulated in G_1 as primary and secondary responses to TGFβ.

TGFβ is involved in a variety of physiological phenomena including immunomodulation, development, angiogeneisis and wound healing (Reviewed in 13). There are three TGFβ homologs in mammals with broadly similar activities *in vitro* (14). TGFβ1 in conjunction with an unrelated hormone, TGFα, permits untransformed fibroblast cells to form colonies in soft agar (15–17). Although originally identified by its growth promoting characteristics, TGFβ has also been shown to be a strong inhibitor of cell proliferation in many cell types and causes

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growth arrest in the G_1 phase of cell cycle (18, reviewed in 14,19).

The mechanism of TGFβ-mediated signal transduction is beginning to be explained. Signaling involves the interaction of two types of receptor (type I and II) both of which are serine/threonine kinases (20). Physiological specificity may be determined by the availability of type I receptors and its substrates. The mechanism by which TGFβ induces cell cycle arrest in some cells is not fully understood, but several connections with known cell cycle signal transduction subroutines have been identified. Most recently, TGFβ has been shown to activate $p27^{Kip1}$ (21), a member of a family of proteins including $p21^{Cip/WAF}$ and others (22–27). This class of proteins inhibits the protein kinase activity of various cyclin dependent kinase (cdk) family members, thereby blocking their phosphorylation of the retinoblastoma gene product and related proteins (28,29). Unphosphorylated or under-phosphorylated Rb is thought to interfere with cell cycle progression by interacting with and inhibiting the activities of several transcription factors, for example E2F. The mechanism by which TGFb activates $p27^{Kip1}$ has not yet been elucidated, but its activity in exponentially growing cells may be regulated by a heat-labile factor, consistent with the fact that the level of $p27^{Kip1}$ protein does not change significantly during cell cycle (21). Ectopic expression of human cyclin A or cyclin E can overcome growth arrest mediated by Rb. TGFβ1 also prevents $p34^{cdc2}$ and $p33^{cdk2}$ proteins from being phosphorylated and thereby converted to their active kinase forms (30,31).

It is interesting to note that TGFβ impacts on the cell cycle regulatory machinery at several points. Through $p27^{Kip1}$, TGFβ potentially blocks cell cycle through interference with all of the major G1 cyclin·cdk functions. TGFβ has also been shown to interfere with the translation of the mRNA for Cdk4, the major cyclin dependent kinase partner of cyclin D (32), which signals through Rb, and is the earliest of this class of genes to be active in G_1 of cell cycle. Cyclin A·Cdk2 activity, which is required for progression through the G1/S boundary, is also blocked by TGFβ through control of the abundance of cyclin A mRNA (33,6). Expression of cdk4 is repressed by TGFβ and constitutive expression of cdk4 can overcome TGFβ-mediated growth arrest (32). Other genes important to cell cycle control and development and a large number of genes involved in the extracellular matrix are also regulated by TGFβ (reviewed 14,19). Not all of these regulatory events channel through Rb (34)

Here we use RAP-PCR to examine the global impact of TGFβ and cycloheximide (CX) on signal transduction pathways and observe genes in various regulatory categories, one of which was not predicted. As a secondary goal, the identification of genes responsive to TGFβ1 treatment is an important step in understanding the physiological role of this factor. In particular, the classification of genes for which expression is regulated by TGFβ as a primary or secondary response is of interest. The response of Mink lung epithelial (Mv1Lu) cells to TGFβ and/or CX was determined during G1 of the cell cycle. We confirm the expectation that the genes currently known to be regulated by TGFβ1 represent only a small fraction of all such genes. Using 14 arbitrary primers or pairs of primers over 1700 RNAs were surveyed during the first few hours after synchronous release from contact inhibition. Eighty-six transcripts whose abundances changed by four-fold or more were observed under the conditions tested. These genes fall into eight different regulatory categories from a total of 27 possible categories.

MATERIALS AND METHODS

Cell culture

Mv1Lu cells (ATCC CCL 64) were grown in MEM alpha medium (GIBCO, Grand Island, NY) supplemented with 10% FBS (14). Confluent cells were split five to one and allowed to reattach for 2.5 hr. Some flasks were treated with 40 pM TGFβ1 (R&D Labs, Minneapolis, MN) and/or with 20 μg/ml cycloheximide (Sigma, St. Louis, Mo) for the periods of time shown in Figure 1. For experiments involving Northern blots in Figure 3 cultures were treated with the TGFβ and/or CX for the times shown in Table 3.

PCR primers

Primers manufactured by Genosys, Woodlands TX, were ZF-8 (5′-CCAGAGAGAAACCCACCA); the reverse sequencing primer RSP (5′-GGAAACAGCTATGACCATGA), N21 (5′-A-CCAGGGGCA); Nu1 (5′-ACGAAGAAGAG) encoding a nuclear localization amino acid motif; Ki1 (5′-GAGGGTGCC-TT) encoding a ser/thr kinase motif; Tp1 (5′-TACATCAACG-C) and Tp2 (5′-CATTGCTCTGC) encoding tyrosine phosphatase motifs; and DD1 (5′-ATGGATGAGGC) and DD2 (5′-GATG-AGGCTGA) encoding the DEAD box motif. Primers from Operon Technologies, Alameda, CA, were N24 (5′-AGGGGC-ACCA), S2 (5′-CCTCTGACTG), S16 (5′-AGGGGGTTCC), S17 (5′-TGGGGACCAC), S19 (5′-GAGTCAGCAG), T10 (5′-CCTTCGGAAG), and U7 (5′-CCTGCTCATC), U11 (5′-A-GACCCAGAG), U12 (5′-TCACCAGCCA).

RAP-PCR fingerprinting using single 18 base primers

RNA was prepared from Mink lung epithelial cells using the guanadinium-CsCl procedure (35). RAP-PCR (3) and differential display (4), is adapted from arbitrarily primed PCR (1,2,36). 10 μl of each RNA at 20, 10 and 5 ng/μl were combined with 10 μl of a 2×reverse transcription mixture containing 100 mM Tris pH 8.3, 100 mM KCl, 8 mM $MgCl_2$, 20 mM DTT, 0.2 mM each dNTP, 10 μM primer (ZF-8 or RSP) and 1 U/μl MuLVRT and ramped from room temperature to 37°C over 5 min., incubated at 37°C for 10 min. and heated to 94°C for 2 min. The final reaction components are 30 mM Tris pH 8.3, 35 mM KCl, 3 mM $MgCl_2$, 5 mM DTT and 5 μM primer. The optimum primer concentration is best determined empirically. After this reaction, 20 μl are combined with an equal volume of a solution containing 10 mM Tris, pH 8.3, 25 mM KCl, 2 mM $MgCl_2$, 0.1 μCi/μl α-[^{32}P]-dCTP and 0.1 U/μl *Taq* polymerase (AmpliTaq, Perkin Elmer, Norwalk, CT), was added and cycled once through 94°C for 5 min, 40°C for 5 min and 72°C for 5 min in a 96-well format thermocycler (Perkin Elmer, CT), followed by 40 cycles through 94°C for 1 min, 60°C for 1 min and 72°C for 2 min. This protocol resulted in the amplification of sequences flanked by arbitrary priming events in both the first and second strand cDNA synthesis steps. 4 μl of each reaction was diluted in 18 μl of 95% formamide, then heated to 94°C for 2 min. 1.5 μl were loaded on a 5% polyacrylamide–50% urea sequencing gel and electrophoresed in 1×TBE at 50 watts for 3 h. The gel was dried and autoradiography performed with X-Omat film (Kodak) for 12 h. The RNA fingerprinting technique described here is covered under a patent application filed on October 15th 1990.

Sequential pairwise RNA fingerprinting using 10 base primers

8 μl of each RNA at 20 and 10 and 5 ng/μl were combined with 8 μl of a 2×reverse transcription mixture containing 100 mM

Tris pH 8.3, 100 mM KCl, 8 mM $MgCl_2$, 20 mM DTT, 0.4 mM each dNTP, 4 μM of the first primer (e.g. N21) and 3.75 U/μl MuLVRT (Stratagene Inc., La Jolla, CA) and ramped from room temperature to 37°C over 5 min, incubated at 37°C for 1 h. and heated to 94°C for 2 min in a 96-well format thermocycler (Perkin Elmer, CT). The reactions were assembled on ice. These 16 μl first strand cDNA reactions were then diluted four-fold to 66 μl with water. 10 μl of this is combined with 10 μl of a solution containing 10 mM Tris pH 8.3, 10 mM KCl, 3 mM $MgCl_2$, 0.4 mM each dNTP, 8 μM second primer (e.g. S2 or U7), 0.1 μCi/μl of α-[^{32}P]-dCTP, 0.4 U/μl *Taq* polymerase Stoffel fragment (Perkin Elmer, Branchburg, NJ) (37). Six different second primers can be used. PCR was performed using 94°C, 30 s; 40°C 1 min; 72°C, 2 min, for 35 cycles. The final reaction components are 18 mM Tris pH 8.3, 18 mM KCl, 3 mM $MgCl_2$, 0.25 mM each dNTP, 0.25 μM first primer (carried over from first strand reaction), 4 μM second primer and 0.2 U/μl *Taq* polymerase Stoffel fragment. Electrophoresis and autoradiography was performed as described above. Virtually all products of reaction using pairwise combinations of primers are distinct from those generated with either primer individually and contain both primers (38,39)

Preparation of clones

Differentially amplified RAP-PCR products were cut from the gel and eluted into 50 μl of TE for 2 h at 65°C. A 5 μl aliquot was then used to reamplify the RAP products employing the same oligonucleotide primers used to generate the fingerprint (40). The RAP-PCR products were cloned into the pMW226 vector, a modified form of the Bluescript vector (Stratagene Inc., San Diego, CA) using standard protocols. For each RAP product six or more independently isolated single stranded phagemids were sequenced using the Sequenase reagent kit v2 (USB, Cleveland, OH) and α-[^{35}S]-dATP (NEN Research Products, Boston, MA).

Southern blots

DNA from RAP-PCR fingerprints were transferred to a membrane (Duralon-UV, Stratagene, La Jolla, CA) by capillary action using 20×SSC buffer and UV cross-linked. Blots were prehybridized with 5×SSC, 0.5% blocking reagent (Boehringer Mannheim Biochemical), 0.8% SDS at 65°C for 4 h. Hybridization to blots used conventional methods (41) and conditions recommended by the vendors. Radiolabeled probes were synthesized by PCR from cloned RAP products using the T7-T3 oligonucleotide primer set (Stratagene Inc., San Diego, CA), and α-[^{32}P]-dCTP.

Northern blots

Poly(A) selected RNA was isolated using the Fast Track mRNA isolation kit (InVitrogen Inc., San Diego, CA). 2.0 μg was electrophoresed through a 1.0% agarose formaldehyde gel (MOPS buffer) and transferred to nylon reinforced nitrocellulose membranes (Stratagene Inc. San Diego, CA). Probes were synthesized by PCR from cloned RAP products using the T7-T3 oligonucleotide primer set, followed by random priming in the presence of α-[^{32}P]-dCTP (Stratagene Inc., San Diego, CA). The G3PDH probe (Clonetech Laboratories Inc., Palo Alto, CA) was radiolabeled using random prime synthesis and α-[^{32}P]-dCTP. Hybridization to blots used conventional methods (41) and conditions recommended by the vendors. Estimates of relative band intensities were determined visually by comparing autoradiographic exposures of different duration to determine the ratio of exposure times that equalized different band intensities.

RESULTS

Tissue culture and RNA fingerprinting

TGFβ1 and its homologs TGFβ2 and TGFβ3 have broadly similar effects on Mink lung epithelial (Mv1Lu) cells, halting cell cycle in G_1 (14). An experiment was designed to determine the proportion of transcripts that are altered in abundance during TGFβ treatment and to divide these transcripts among the primary response class (which do not need new protein synthesis in order to be regulated) and secondary response classes (which do need new protein synthesis). These classes can be distinguished by using cycloheximide (CX), an inhibitor of protein synthesis. In addition CX is known to stabilize some otherwise rapidly degraded RNAs (7,8) and induce transcription of other genes (9−12).

Mv1Lu cells were released from growth arrest due to confluence by reseeding at lower density and allowed to reattach for 2.5 hours in media containing serum. Cells were then treated with TGFβ1, CX or both and were harvested at the times indicated (Figure 1). The expected growth arrest response to TGFβ was confirmed by monitoring cell division (18).

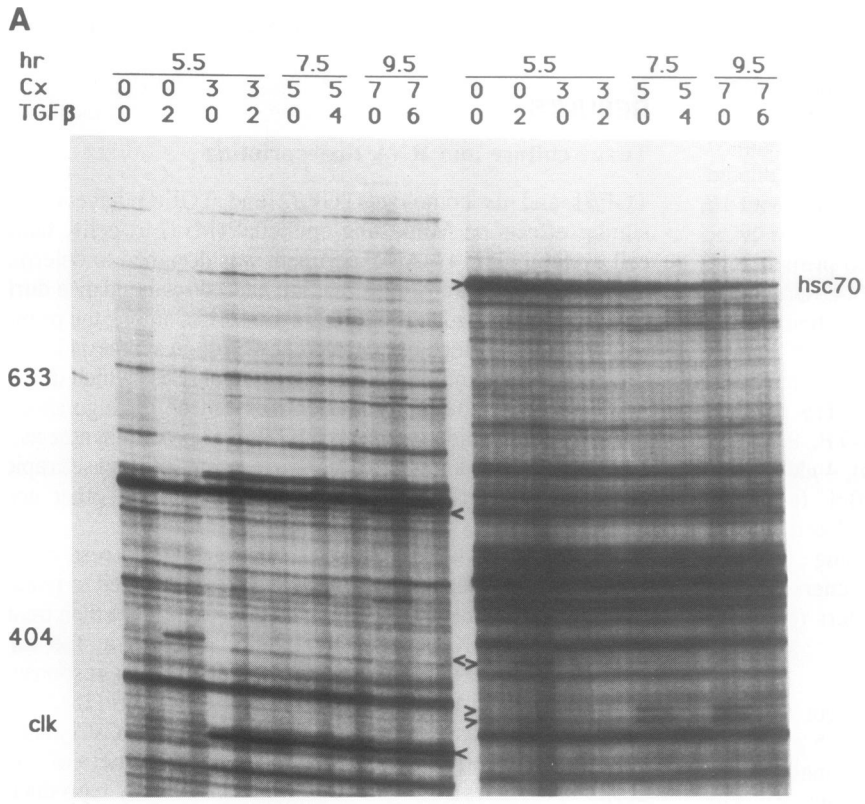
Total RNA was prepared by the guanadinium/CsCl method (35). RNA fingerprints were generated using primers of either 10, 11 or 18 bases in length, all of which give highly reproducible and complex fingerprints. The fingerprints were resolved on denaturing polyacrylamide gels, as described in the methods. In Figure 1, several examples of RAP-PCR fingerprints, are shown with some of the differentially amplified products indicated by arrows.

When using RAP-PCR, it is important to control for differences in RNA quality and concentration. Otherwise, some of the differentially amplified products are likely to reflect concentration or quality differences rather than alterations in abundance. To control for these effects, each RNA was fingerprinted at three two-fold serial dilutions and these fingerprints were loaded side-by-side. Only those differentially amplified products that were reproducibly present or absent at all three RNA concentrations were considered to be derived from differentially expressed genes.

The primers used for RAP-PCR included some that encoded short conserved amino acid motif sequences such as part of the ser/thr kinase motif (listed in Materials and Methods). Other researchers have shown that RAP-PCR fingerprinting using primers encoding extensive motifs can bias towards members of particular gene families (42). We do not yet have evidence that the same is true for shorter motif sequences encoded in 10 or 11 bases.

The number of regulatory categories surveyed

Consider only three combinations of only two treatments; TGFβ-treated, CX-treated, and TGFβ+CX. Information can be obtained regarding the level of gene expression in 27 possible categories [3^3] (Table 1). As the number of treatments increases the number of possible regulatory categories increases exponentially. Three treatments can be used in seven possible combinations, increasing even further the information about each sampled gene; 2187 [3^7] possible regulatory categories result from using all seven combinations of modulators. If quantitative


A
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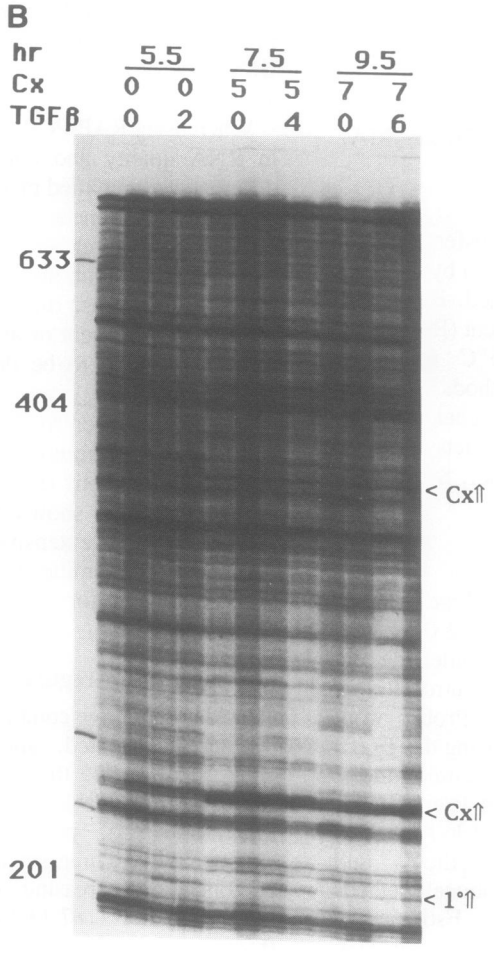
Cx
TGFβ
5.5
7.5
9.5
0 0 3 3 5 5 7 7
0 2 0 2 0 4 0 6
hsc70
633
404
clk


B
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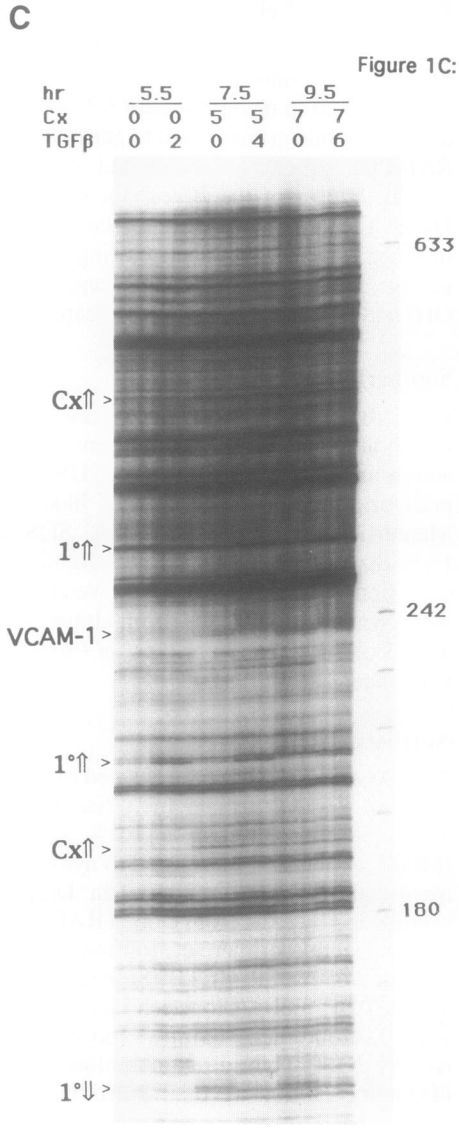
< 1°⇑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1°⇑ >
VCAM-1 >
1°⇑ >
Cx⇑ >
1°⇓ >
242
180

Figure 1C:

changes in gene expression are also considered then the number of categories increases further. Information is provided not only by the occupied categories but also by which regulatory categories are unoccupied.

To illustrate these concepts we choose the simplest case of two modulators and twenty-seven possible regulatory categories. Using 14 arbitrary primers over 1700 RNAs were surveyed during the first few hours after synchronous release from contact inhibition. Eighty-six transcripts whose abundances changed by four-fold or more were observed under the conditions tested (Table 2). These genes fell into eight different regulatory categories. Most transcripts fall into the trivial first category:

Table 1. Three treatments yield twenty-seven possible response categories

	I	C	I+C
1	0	0	0
2	⇑	0	0
3	⇓	0	0
4	0	⇑	0
5	0	⇓	0
6	0	0	⇑
7	0	0	⇓
8	⇑	⇑	0
9	⇑	⇓	0
10	⇕	0	⇕
11	⇑	0	⇓
12	⇓	⇑	0
13	⇓	⇓	0
14	⇓	0	⇑
15	⇓	0	⇓
16	0	⇑	⇕
17	0	⇑	⇓
18	0	⇓	⇑
19	0	⇓	⇓
20	⇑	⇑	⇑
21	⇓	⇓	⇓
22	⇓	⇑	⇑
23	⇑	⇓	⇑
24	⇑	⇑	⇓
25	⇑	⇓	⇓
26	⇓	⇑	⇓
27	⇓	⇓	⇑

(0 = no change; ⇑ = increased expression; ⇓ = decreased expression). The shaded response categories were observed in our experiments. One major caveat is that 'no change' includes cases where the transcript was not observed in both the control and the treatment in question.

Up-regulated as a primary response

The abundances of ten transcripts were increased in the presence of TGFβ and were not affected by CX. (Class 10 in Table 1). These genes join a handful of others in the TGFβ primary response category including plasminogen activator inhibitor-1 (PAI-1) and urokinase-type PA (uPA) (43), jun B (44), fibronectin and alpha 2(I) procollagen (45), actin (46) and TI 1 (47).

There were at least three examples of genes in class 5 in Table 1 for which TGFβ seems to overcome the ability of CX to down regulate the transcript although the gene is not detectable when TGFβ is used alone. These genes are included in the primary response category for convenience because this TGFβ effect occurs in the presence of CX.

Secondary response

The abundances of nine transcripts increased upon TGFβ treatment but this effect was blocked in the presence of CX, indicating that they corresponded to secondary response genes (Class 2 in Table 1). Genes controlled by a labile activator would also fall in this class and connot be distinguished from secondary response genes at our level of analysis.

The transcripts in this class accumulate detectably over two hours and are therefore biased somewhat towards the fastest responding subset among the secondary response genes. There are presumably other RNAs that accumulate more slowly and were not surveyed in this particular protocol. For example, we have shown that RAP-PCR detects the four-fold up-regulation of RNA for the extracellular matrix protein osteonectin after 24 hrs of TGFβ treatment (6) though we do not know if this occurs as a secondary response. A previously identified gene that is up-regulated by TGFβ as a secondary response is the alpha 1(IV) collagen gene (48).

Only one gene was down regulated by TGFβ as a secondary response (class 3, Table 1). It is probable that some transcripts that are down-regulated in the presence of CX or TGFβ have much longer half lives and would have been difficult to detect in these experiments. Transcripts that are down-regulated by TGFβ as a secondary response (or controlled by a labile repressor), have been observed before (e.g. 49). We have previously shown that this response class is probably occupied by a large number of genes after 24 hrs of TGFβ treatment, including TRT311-1 and cyclin A (6).

Down-regulated by cycloheximide

Sixteen transcripts appeared to decline during CX treatment. TGFβ had no effect on the level of these transcripts (Class 19 in Table 1). CX has previously been reported to down-regulate only a few genes at the transcriptional level including, for example, insulin-like growth factor-binding protein-1 (50).

Up-regulated by cycloheximide

The abundances of forty-seven transcripts increased in response to CX treatment. The steady state levels of many known transcripts, including several oncogenes, increase in the presence of CX due to the inhibition of mRNA degradation rather than an increase in transcription (reviewed in 7,8). The mechanisms controlling the abundances of the forty-seven transcripts observed here are yet to be established.

A new intersection between regulatory categories

The final category of differentially expressed transcripts included ten of the forty seven transcripts whose abundances increased in response to CX treatment. These ten transcripts were suppressed by concurrent treatment with TFGβ and are in class 4 (or perhaps class 26) in Table 1. The balance of 37 genes were unaffected by TGFβ (Class 16 in Table 1). The ability of TGFβ to repress induction by CX can be thought of as primary down-regulation, a class that was both unexpected and large. The observation of this class in an experiment involving a relatively small number of different treatment groups emphasizes the ease

Figure 1. RAP-PCR fingerprints. Confluent cells were reseeded and harvested 5.5, 7.5 and 9.5 hours later. Some cells were treated with Cx 2.5 h after reseeding and/or TGFβ 3.5 h after reseeding. Such cells experienced Cx treatment for 3, 5, or 7 h and/or TGFβ treatment for 2, 4, or 6 h before harvesting. RNA was prepared from these eight sets of treatment regimes as described in the methods. 160, 80 and 40 ng of total RNA was fingerprinted, of which only two concentrations are shown. Separation was on a 5% polyacrylamide−50% urea sequencing gel with electrophoresis in 1×TBE at 50 watts for 3 h. The gel was dried and autoradiography performed with X-Omat film (Kodak) for 12 h. Some differentially amplified cDNAs are indicated by arrows. Panel **A** is experiment 407 with primer RSP on the right, ZF-8 on the left; **B** is expt 429 with primer N21 and S2; **C** is expt 429 with primer N21 and U7. Each image shows only a portion of the data. Single stranded DNA markers are indicated in bases.

with which it should be possible to discern previously unknown interactions transcript modulation mechanisms.

Further characterization of differentially amplified products

The cloning of transcripts is not necessary to estimate the proportion of transcripts that respond to a treatment or to observe new regulatory interactions between modulators of RNA abundance. However, for the following reasons seven transcript fragments were cloned in these studies. First, the technology is still new enough that confirmation by Northern blot is desirable to demonstrate that the observed differentially amplified products truly represent differentially expressed genes. Second, some of the transcripts were likely to be in the database. If these genes were already known to occupy the response category we observed then this information would constitute independent validation of the efficacy of the treatment conditions. This criterion does not require that the cloned transcript encode an important component in the response to a modulator but merely that it is a known target of the response. Third, if the regulation of some of the genes identified has already been studied but not under the conditions we used then, when new interactions are observed, additional information on regulation of the gene will be acquired. Fourth, the proportion of potentially new genes discovered would give a rough estimate of the number of genes still to be characterized in each response category. Finally, if the clones were shown to be from independent transcripts this would support arguments based on the probability that the genes are sampled in an unbiased manner from the pool of RNAs (excluding rare transcripts).

All differentially amplified products in Table 2 were isolated from the gel and reamplified using the same primer(s). Each fragment was named using the acronym 'TRT' for TGFβ-regulated transcript, or 'CRT' if only CX-responsive, followed by the experiment number and a unique number assigned to each transcript.

One or more examples of differentially amplified products in five of the eight observed regulatory categories were selected for cloning. The reamplified fragments were blunt end ligated into pCRscript (Stratagene, La Jolla). Sequences were obtained from six or more phage-mobilized ssDNA for each differentially amplified product and the most common sequence was used as a probe against a Southern blot of the original gel to confirm that the correct fragment had been cloned rather than one of the other unregulated products that run in the same place in the gel (examples presented in Figure 2). About 75% of cloning attempts yielded clones that occurred more than once in six sequences. These clones gave positive Southern blots to the correct fragment in 80% of cases. The sequences of seven clones confirmed in this manner were deposited in the GenBank database (U13884–U13891).

All sequences were compared to the GenBank DNA and protein databases. While the DNA comparison is straightforward, the comparison of conceptually translated sequences is complicated by the fact that the sense orientation of some of the gene fragments was unknown and the fact that many fragments can be expected to derive from non-coding regions of the transcript. The sequences were conceptually translated in all six frames and compared to the protein database using a weighted match matrix designed to allow the detection of distant family members (51).

The orientation of the sense strand was known for some products because when a single primer is present during first strand cDNA synthesis and a second primer is added for the second strand synthesis, as in Figure 1B and 1C, the first primer generally occurs at the 3′ end of the product relative to the mRNA and second primer generally defines the 5′ end (38,39). Thus, the sense strand is known in all such cases. For these clones only open reading frames (ORFs) in the correct orientation need be considered. Thus an ORF in the wrong orientation in TRT429-4.5 (GB U13886) could be rejected as a chance event whereas an ORF in the correct orientation in TRT429-5 (GB U13885) may have greater significance.

Of the seven regulated genes confirmed by Southern and Northern blots, all proved to be from different independent transcripts. Four transcripts were apparently novel. Of the three previously known transcripts, two were in the expected response categories and one had no previously known regulator. The genes cloned were: (1) One novel up-regulated primary response gene, TRT429-5. (2) One novel secondary response gene, TRT407-1. (3) One clone contained the open reading frame for *hsc*70 (GB U13889) a chaperone that helps in protein folding. This transcript declined dramatically during 6 hrs of CX treatment. The mRNA for this gene, *hsc*70 is known to have a very short half life (52). Perhaps *hsc*70 is down-regulated when there are no nascent proteins to be folded. A time course of treatment was also incorporated into our experimental design. The trajectory of the *hsc*70 regulatory response is seen in Figure 2C. The trajectory of the response is also visible for genes in the other regulatory categories and these trajectories sometimes differ within a response category. See, for example, Figure 2A and 2D where the inhibition by TGFβ wanes more quickly in 2D than in 2A. (4) One clone for an CX-induced transcript contained an open reading frame from *clk*, a member of the *cdc*2 gene family (53,54, GB# U13890). CX-responsiveness and TGFb unresponsiveness are the first two known phenotypes for this gene. (5) Among the unexpected CX-inducible, TGFβ-repressible class two apparently novel transcripts (TRT407-2 and TRT407-9 were cloned and verified by Northern analysis (Figure 3A and 3D). The corresponding RAP-PCR products are largely absent from the control (minus CX) lanes, indicating that their abundances are much lower in cells that have not been treated with CX. The possibility remains, however, that some or all of these genes are also down-regulated in the presence of TGFβ alone.

A third clone in this category proved to be vascular adhesion molecule-1 (VCAM-1). The match with VCAM-1 was in the 3′ non-coding region. This gene is involved in cell–cell adhesion and is known to be expressed in both endothelial and epithelial cell types, where its physiological role includes facilitation of lymphocyte migration to sites of inflammation. Concordant with our data, VCAM-1 can be superinduced by CX in renal tubular epithelial cells (55) and is down-regulated by TGFβ in some cell types (56,57) including other epithelial cells but not endothelial cells (58,59). A TGFβ-mediated decrease probably also occurs in Mv1Lu cells in the absence of CX, although we were not able to detect this because the product was too faint in uninduced cells both in RAP-PCR fingerprints and in Northern blots.

Among the clones for which no match could be found in the databases, some may derive from divergent non-coding or unsequenced portions of genes or gene family members already known in mink or other species. Others may be entirely novel, although proof of this will require sequencing of larger clones derived from screening a full length cDNA library. Among the potentially novel genes, TRT429-5 (GB# U13885) is of

Table 2. Differentially amplified transcripts identified by RAP-PCR

cDNA Name	Gene Identity	Response Class	RAP: Early G1 Control	TGFβ	CX	TGFβ+CX
			2hr	2	2, 4, 6	2, 4, 6[a]
TRT429-1.5		1°↑	0[b]	+	0	+
TRT429-3		1°↑	0	+	0	+
TRT429-4.2		1°↑	0	+	0	+
TRT429-5	new[c]	1°↑	0	+	0	+
TRT429-6.2		1°↑	0	+	0	+
TRT474-6		1°↑	0	+	0	+
TRT474-9.5		1°↑	0	+	0	+
TRT474-14		1°↑	0	+	0	+
TRT475-2		1°↑	0	+	0	+,++
TRT475-3		1°↑	++	++	+	++
TRT476-15		1°↑	++	++	0,+	++,++
TRT476-16		1°↑	+	0	+	0
TRT477-1		1°↑	0	+	0	+
TRT477-3		1°↑	+	+	0	+
TRT407-2	new	1°↓	0	0	+	0
TRT407-9	new	1°↓	0	0	+++	0,+,++
TRT407-10		1°↓	0	0	++	+
TRT429-4.5	VCAM-1	1°↓	0	0	+	0
TRT429-6.5		1°↓	0	0	+	0
TRT474-9.2		1°↓	0	0	+	0
TRT476-5		1°↓	++	+	+++	+,++
TRT476-6		1°↓	++	+	++,+++	+,++
TRT476-16		1°↓	+	0	+	0
TRT477-3		1°↑	+	0	+	0
TRT407-1	new	2°↑	0	+	0	0
TRT407-7		2°↑	0	+	0	0
TRT475-6		2°↑	0	+	0	0
TRT474-1		2°↑	0	+	0	0
TRT476-4		2°↑	0	+	0	0
TRT476-17		2°↑	0	+	0	0
TRT477-0.2		2°↑	0	+	0	0
TRT475-4		2°↓	+	0	+	+
CRT407-6	clk	Cx↑	0	0	+	+
CRT429-0		Cx↑	0	0	+	+
CRT429-1		Cx↑	0	0	+	+
CRT429-2		Cx↑	0	0	+	+
CRT429-4		Cx↑	0	0	+	+
CRT429-5.5		Cx↑	0	0	+	+
CRT438-1		Cx↑	0	0	+	+
CRT438-2		Cx↑	0	0	+	+
CRT438-6		Cx↑	0	0	+	+
CRT438-7		Cx↑	0	0	+	+
CRT438-8		Cx↑	0	0	+	+
CRT438-9		Cx↑	0	0	+	+
CRT474-4		Cx↑	0	0	+	+
CRT476-7		Cx↑	0	0	+	+
CRT474-8		Cx↑	0	0	+	+
CRT474-9		Cx↑	0	0	+	+
CRT474-10		Cx↑	0	0	+	+
CRT474-12		Cx↑	0	0	+	+
CRT474-15		Cx↑	0	0	+	++
CRT474-16		Cx↑	0	0	+	+
CRT475-1		Cx↑	0	0	+,++	+,++
CRT475-5		Cx↑	0	0	+	+
CRT476-1		Cx↑	0	0	+,++	+,++
CRT476-2		Cx↑	0	0	+	+
CRT476-7		Cx↑	0	0	+	+
CRT476-8		Cx↑	0	0	+	+
CRT476-11		Cx↑	0	0	+	+
CRT476-12		Cx↑	0	0	+	+
CRT476-13		Cx↑	0	0	+	+
CRT476-14		Cx↑	0	0	+	+
CRT476-18		Cx↑	0	0	+	+
CRT476-19		Cx↑	0	0	+	+

Table 2. (*cont.*)

CRT476-22		Cx↑	0	0	+	+
CRT476-23		Cx↑	0	0	+	+
CRT477-0.5		Cx↑	0	0	+	+
CRT477-0.7		Cx↑	0	0	+,0	+,0
CRT477-5		Cx↑	0	0	+	+
CRT477-5.5		Cx↑	0	0	+	+
CRT407-8	hsc70	Cx↓	+++	+++	+++,++,+	+++,++,+
CRT438-3		Cx↓	++	++	+	+
CRT438-4		Cx↓	++	++	+	+
CRT438-5		Cx↓	++	++	+	+
CRT474-2		Cx↓	+	+	+,0	+,0
CRT474-3		Cx↓	+	+	0	0
CRT474-5		Cx↓	+	+	0	0
CRT474-11		Cx↓	+	+	0	0
CRT474-13		Cx↓	+	+	0	0
CRT476-3		Cx↓	++	++	+	+
CRT476-9		Cx↓	+	+	0	0
CRT476-10		Cx↓	+	+	0	0
CRT476-20		Cx↓	+	+	0	0
CRT476-21		Cx↓	+	+	0	0
CRT477-2		Cx↓	+	+	0	0
CRT477-4		Cx↓	+	+	0	0
CRT477-7		Cx↓	+	+	0	0
			24hr	24hr		
TRT311-1[d]	new	?	+	0		
TRT311-2[d]	cyclin A	?	++	+		
TRT311-3[d]	osteonectin	?	+	++		

[a]Times of CX and TGFβ+CX treatment were 2 and 6 hours in experiments 474, 475, 476, 477.
[b]'0' indicates undetectable. +, ++, and +++ indicate increases in the relative levels of detectable product over time. The relevance of the number of pluses does not extend to comparisons between different RAP-PCR products where '+' may indicate either a prominent or a weak RAP-PCR product.
[c]'new' indicates not in database.
[d]Information from Ralph *et al.*, 1993 is not included in the cumulative data in Table 4 because of differences in protocol.

particular interest because an ORF in the sense strand that spans the RAP-PCR fragment is not closely related to any known protein.

Northern blots

The Northern blot data is summarized in Table 3. In our experience, virtually all cloned RAP-PCR products that show the correct distribution on Southern blots of RAP-PCR fingerprints and that give detectable signals on Northern blots are found to be differentially expressed in the expected manner (e.g. 3,60,6,61). Furthermore, the difference in intensity of a particular differentially amplified product between samples generally corresponds to the actual ratio of the RNA abundances (3,60,6, and experiments described here). The reason for the extraordinary and useful stoichiometry between RAP-PCR fingerprint lanes is that, unlike ordinary PCR amplification of a single discrete product, the mass of a differentially amplified product is only a very small fraction of the total amplified mass which includes other, generally invariant, PCR products. Thus, the effective number of amplification cycles for each individual PCR product is nearly identical between the different RNA samples and the ratio of amplification for each PCR product reflects the ratios of the starting materials. Obviously, such arguments only apply when almost identical patterns are obtained using different template concentrations as presented in Figure 1.

In the preparation of RNA for Northern blots, conditions were chosen that would further illuminate the regulation of each gene while still catching any erroneous clones that might have slipped through the screening process. RNA was prepared from cells growing synchronously in G_1 or from confluent cells. In each case cells were treated with TGFβ, CX, or both, in a manner similar to that used for the RAP-PCR experiments presented above. Poly(A)+ selected RNA was prepared for the Northern blots because most of the transcripts examined were too rare to be detected otherwise. Indeed, based on the exposure time necessary for transcript detection, the G3PDH mRNA control was of at least 1000-fold greater abundance than most of the clones tested. Clones 407-1 and 429-5 yielded positive Southern blots but failed to give positive Northern blots, presumably because the RNAs are of very low abundance. Among the genes that gave positive signals on Northern blots, all were independent transcripts, indicating that most of the amplified products in each fingerprint are from independent transcripts.

All the clones that gave signals on Northern blots had similar distributions of expression as seen by RAP-PCR for synchronous cells (e.g. Figure 3A and 3B). However, there was one exception for confluent cells, TRT407-9 (Figure 3D). The repressive effect of TGFβ on this transcript seems to wane in G_1 and logarithmically growing cells after a few hours, whereas the gene remains more fully down-regulated by TGFβ in confluent cells.

The reduction in *hsc*70 RNA abundance observed in Figure 1 could occur as the cell advances through G_1 or it could be caused specifically by CX treatment. To distinguish between these possibilities we determined the changes that occur in the RNA level for this gene during G_1 in the absence of CX and in confluent cells. These data, in Figure 3C and summarized in Table 3 show that the steady state level of this RNA is down-regulated in the presence of CX but not in untreated confluent

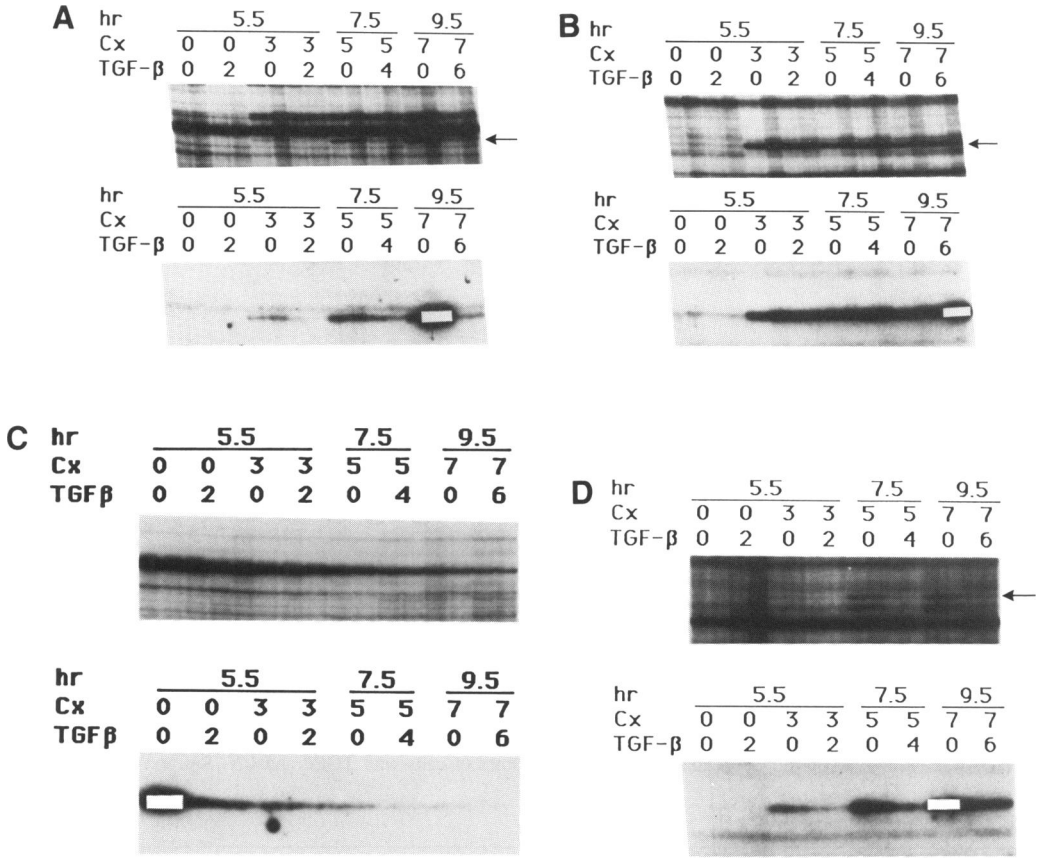

Figure 2. Southern blots of RAP-PCR fingerprints. The RAP-PCR gel was transferred to a nylon membrane and probed with a radiolabeled clone. See Methods for Details. The top panel in each example shows the RAP-PCR gel. The bottom panel show the Southern hybridization. The clones used as probes in these examples are: Panel **A**, TRT407-2; **B**, CRT407-6, *clk*; **C**, CRT407-8, *hsp70*; **D**, TRT407-9.

cells or in untreated cells in G_1. Whether CX-mediated repression occurs at the RNA stability or transcriptional level is still to be determined.

DISCUSSION

Intersection among regulators of transcript abundance

A great strength of RNA fingerprinting by arbitrarily primed PCR is that many RNAs can be compared in parallel, such that the effects of a number of modulators of RNA abundance can be examined simultaneously. By comparing a large number of different RNA preparations from cells that have experienced different treatments, previously unknown interactions between signal transduction pathways can be observed. Signal transduction pathways shared by different modulators will appear as an intersection in the set of transcripts they influence. This approach takes advantage of fingerprints as highly information rich molecular phenotypes for transcript regulation. Much can be learned about the interactions between modulators of RNA abundance even when the responsive transcripts remain anonymous. Discovering new regulatory categories or identifying the proportion of genes that are altered by a particular treatment are more difficult to achieve by subtractive hybridization, differential screening or two dimensional protein gel electrophoresis where each pairwise comparison must be performed separately (e.g. 47).

We choose to examine the simplest case in which two modulators can be used to investigate twenty-seven possible regulatory categories (Table 1). The response of Mink lung epithelial (Mv1Lu) cells to TGFβ and/or CX was determined during G1 of the cell cycle. Over 1700 RNAs were surveyed during the first few hours after synchronous release from contact inhibition, using 14 arbitrary primers. Eighty-six transcripts whose abundances changed by four-fold or more were observed. Genes were observed in eight of the 27 regulatory categories.

The most interesting observed category was found among the forty-seven CX up-regulated transcripts. Ten of these transcripts remained TGFβ repressible in what we call a primary down regulatory response. CX is known to stabilize some mRNAs by overriding a sequence-specific RNA degradation pathway (reviewed in 7,8) and CX treatment induces other genes at the transcriptional level, in some circumstances (9–12) even at levels of CX that are too low to inhibit protein synthesis significantly (62). TGFβ could down-regulate a subset of CX up-regulated RNAs either by triggering transcriptional repression or by overriding the CX effect on mRNA stability. Cycloheximide and puromycin inhibit different steps in protein synthesis and often have different effects on RNA transcription and stability (e.g. 10). Experiments using puromycin and CX alone or together will allow the further dissection of the CX-responsive classes of genes. Other ways of dissecting the various response classes can be envisioned, such as treatment with the transcriptional inhibitor,

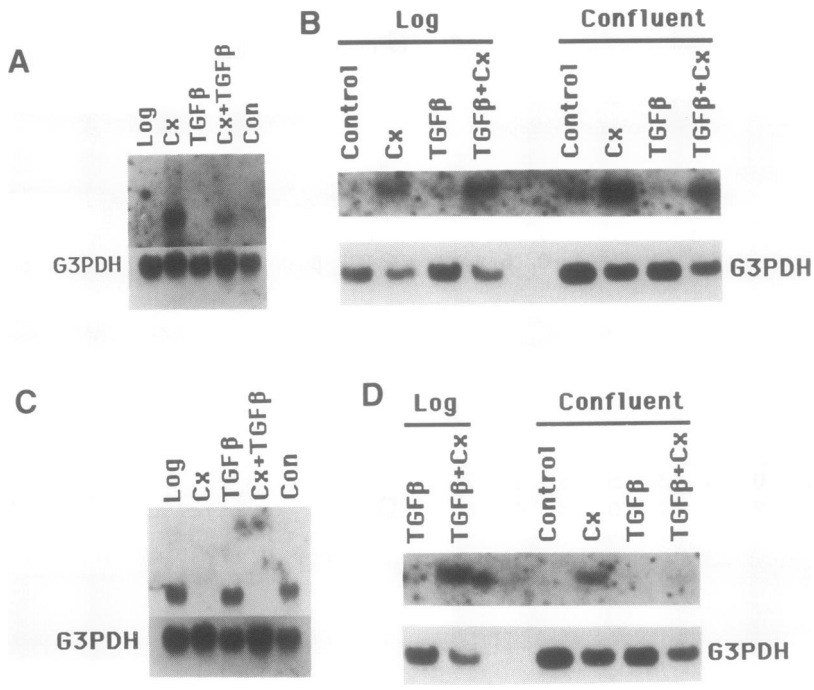

Figure 3. Northern blots. The clones used as probes are Panel **A**, TRT407-2; **B**, CRT407-6, *clk*; **C**, CRT407-8, *hsp*70; **D**, TRT407-9. The glyceraldehyde-3-phosphate-dehydrogenase gene (G3PDH) was used as a control. See Table 3 for incubation times of cells with TGFβ and CX. See text for details.

Table 3. Northern blot data

cDNA Name	Gene Identity	Response Class	RAP: Early G1 Ctrl 2hr	RAP: Early G1 TGFβ 2	RAP: Early G1 CX 2, 4, 6	RAP: Early G1 TGFβ+CX 2, 4, 6	Northern: Early G1[a] Ctrl 7	Northern: Early G1[a] TGFβ 6	Northern: Early G1[a] CX 7	Northern: Early G1[a] TGFβ+CX 7	Northern: Confluent Ctrl 0	Northern: Confluent TGFβ 6	Northern: Confluent CX 7	Northern: Confluent TGFβ+CX 7
TRT407-1	new	2°↑	0[b]	+	0	0		0[c]						
TRT407-2	new	1°↓	0	0	+	0	0	0	+++	+	0			
TRT407-9	new	1°↓	0	0	++	0,+,++			+	+	0	0	+	0
CRT407-6	clk	Cx↑	0	0	+	+	0	0	+	+	0	0	+	+
CRT407-8	hsc70	Cx↓	+++	+++	+++,++,+	+++,++,+	+	+	0	0	+			
TRT415-1	new	2°↑	0	+			0	++	+	+	0			
TRT429-6.5	VCAM-1	1°↓	0	0	+	0			++	0				

[a]Treatment times indicated are measured starting 2.5 hrs after reattachment of reseeded confluent cells. TRT415-1 was studied using RNA from asynchronous cultures treated for the times indicated.
[b]'0' indicates undetectable. +, ++, and +++ indicate increasing levels of detectable product over time.
[c]Perhaps not detectable because of low abundance.

actinomycin, which would reveal which genes are regulated at the level of transcription versus RNA stability.

The substantial antagonistic intersection observed here between TGFβ and CX was not predicted but suggests new hypotheses: transcriptional responses that take place on a scale of a few hours must generally involve transcripts that are potentially unstable, allowing for their rapid removal at the appropriate time. CX is known to stabilize many such transcripts. Thus, we hypothesize that a substantial fraction of TGFβ-repressible genes can be regulated by a mechanism that is affected by CX. Furthermore, transcripts that are stabilized by CX are potentially a rich source of candidates for modulation by other hormonal treatments that may require the rapid removal of labile transcripts. We predict that there will be a considerable intersection between the transcripts in the CX up-regulated category and transcripts that respond rapidly to many hormones and other natural modulators of transcript abundance. RAP-PCR of biological samples treated with CX and treated with other modulators of RNA abundance could be a widely applicable means to observe the effect of modulators on the particularly interesting subset of transcripts that can be rapidly turned over. Thus, despite the fact that CX is a poison that is unlikely to have an exact biological counterpart, this drug has been invaluable in parsing genes into regulatory classes and may have identified a subset of transcripts in the cell that are critically involved in the rapid response to natural modulators.

The identification of interactions among modulators of transcription and the resulting partial dissection of the regulatory pathways which this implies does not require sequence information. However, such sequence information is critical to understanding the orchestration of TGFβ- and CX-induced physiological responses. Ultimately, it will be valuable to compare the promoters and other *in cis* regulatory elements for genes that are in the same or similar regulatory classes because these structures will provide information on the molecular mechanism of the regulatory class. Also, reverse genetics

Table 4. Size of TGFβ and CX response classes

Experiment number	Primer name(s)	Minimum Survey Size	TGFβ 2°↑	TGFβ 2°↓	TGFβ 1°↑	Cx 1°↓	Cx ↑	Cx ↓
407	RSP	79	2	0	0	3	1	1
	ZF-8	73						
429	N21 & S2	155	0	0	5	2	5	0
	N21 & U7	154						
438	N21 & Nu1	80	0	0	0	0	6	3
	N21 & Ki1	75						
474	S19 & S17	110						
	U12 & U11	152	1	0	3	1	8	5
	N24	133						
475	S19 & S16	107	1	1	2	0	2	0
476	S19 & Tp1	64						
	T10 & Tp2	120	4	0	1	3	12	4
	U12 & Ki1	104						
	N24 & DD1	103						
477	S19 & Nu1	119	1	0	2	1	3	3
	N24 & DD2	120						
Total cDNAs		1748	9	1	13[a]	10	37	16
Class size (%)[b]		100	0.5	0.01	0.7	0.6	2.1	0.9

[a]Includes class 5 and 10 in Table 1.
[b]Upper limit for up-regulated transcripts and lower limit for down-regulated transcripts. Rare transcripts are likely to be under-represented in this sample.

experiments in which the proteins encoded by the genes we have identified are either over-expressed or repressed will allow the exploration of the role of these genes in the response to transcript modulators.

The magnitude of the TGFβ and cycloheximide responses

RAP-PCR fingerprints represent a sample of all transcripts except that rare transcripts are probably under-represented (5,6). Thus, it is possible to extrapolate to the approximate proportion of transcripts that respond to a particular treatment. Such calculations are easy to make, as we show here, and even the crudest estimates of the number of regulated transcripts have not been made previously for most modulators of RNA abundance. Indeed, much of what is known about the overall scale of differential gene expression is derived from ad hoc comparisons of information for individual genes or from two dimensional protein gel electrophoresis (e.g. 63). This latter method observes only the most abundant proteins and their modifications. Furthermore, characterization of differentially expressed proteins requires comparison of two or more separate 2D gels followed by micro-sequencing of well isolated protein spots. Nevertheless, this strategy has been used extensively and atlases of such information have been compiled (e.g. 64). 2D protein gels can be used to assay protein levels, modification and stability, so RAP-PCR complements rather that replaces these methods.

We have estimated the magnitude of the response of Mv1Lu cells to TGFβ and CX in 27 possible regulatory categories of which eight categories were observed. A sample size of 1,748 cDNAs was estimated by counting the PCR products in the control lane for which we would be likely to detect a four-fold reduction of intensity if it occurred. Extensive experience with cloning of fragments and hybridization back to a Southern blot of the original RAP-PCR gel indicates that virtually all fragments are independent of each other. To the extent that the transcripts we sampled constitute an unbiased representation of the total RNA population we can estimate the proportion of genes in each response category, as presented in Table 4. Given the proper controls (high reproducibility of the fingerprints at two RNA concentrations), it is unnecessary to clone genes to perform these calculations. Nevertheless, some genes were cloned and confirmed to be appropriately regulated (Results).

Setting aside for a moment the issue of abundance normalization (the frequency of sampling rarer RNAs), these data indicate that in the first few hours of G_1 the abundances of as many as 0.7% of transcripts may increase as a primary response to TGFβ. As many as 2.7% of transcripts may increase during CX treatment, of which one third do not increase when TGFβ is also present. These numbers reflect changes in RNA levels that, in some cases, are as little as four-fold up or down, although some changes are much greater.

Estimates of the size of each response class presented in Table 4 must be considered upper limits for those response classes that involve increases in the steady state level of RNA. This is because the total number of PCR products surveyed also includes an unknown number of transcripts that would have been visible had their abundances increased. The number of products actually surveyed will be much larger than the observed visible products.

Conversely, the sizes of the response classes that are down-regulated during the course of the experiment can only be surveyed among the visible products, a number which is accurately known. However, the number of transcripts that are down-regulated may be systematically under-estimated because the abundances of these RNAs may decline slowly relative to the duration of the experiment.

The likelihood that any particular response class is represented in a fingerprint depends on the sequence complexity of the class and the abundance of its members. The interplay between complexity and abundance in RAP-PCR is not entirely clear. Due

to the abundance normalization problem, RAP-PCR fingerprints may not reflect the behavior of the total RNA population. Nevertheless, the scale of the response that we can calculate is probably accurate except among the rarest RNAs and it is entirely possible that the rarer RNAs respond to a similar extent.

In these experiments we observed eight of the 27 possible regulatory categories (including the trivial 'unchanged' category). Some of the empty categories probably correspond to regulatory categories that are not used. However, scenarios for some regulatory phenomena that were not observed can easily be envisioned. For example, transcripts that are up-regulated by TGFβ only in the presence of CX (class 6, Table 1) could include genes that are only transiently expressed in the presence of TGFβ and for which the mRNA would be degraded unless CX was present. Representatives of such regulatory classes may not have been identified because the time course used in this experiment may exclude most members of such classes of genes. A few categories may appear empty because all of the responsive transcripts were of too low an abundance to be sampled (see refs. 5,6 for why transcripts are underrepresented). Alternatively, categories that contain few regulated genes are likely to seem empty in a limited sample. For example, a class representing 0.1% of all the genes that can be sampled using this method has an 18% probability of not being sampled in a survey of 1,700 transcripts. However, there is about a 99.99% chance that at least one transcript will be sampled in a category containing 0.5% of all genes (excluding low abundance transcripts) [$0.995^{1,700}$]. Thus, we can be almost certain that none of the 19 seemingly empty categories have responsive transcripts representing more than 0.5% of all assayable transcripts.

In a sense a calculation of the scale of differential gene expression is an extension of the hybridization kinetics work of the 1960's and 70's (e.g. 65). However, with RAP-PCR many different RNA samples can be compared simultaneously. Because the complex molecular phenotype generated by RAP-PCR reflects changes in abundances of hundreds of individual RNAs, hypotheses regarding the interactions of signal transduction pathways can be tested and new hypotheses generated, for example, by comparing the effect of different combinations of hormones and drugs on differential gene expression. One unconventional conclusion is that the transcripts assayed in RAP-PCR fingerprints can remain anonymous during this process, although, genes from assorted regulatory categories can be cloned directly as needed. Indeed, assuming that there are 15,000 expressed genes in the cell, then fragments from more than 10% [$1 - e^{-(1,700/15,000)}$] of genes that are up-regulated in the first few hours by either TGFβ or CX have been identified using only 14 primers or primer pairs.

Ultimately, the examination of RNAs from cells exposed to different treatments in parallel could allow a logical and parsimonious network of observed regulatory categories to be developed and integrated with information acquired by other methods. The data in Table 4 can be integrated into such a network. As more data is acquired such a network could be useful as a predictive tool for future experiments and could be of a complexity rivaling that of the familiar schematics outlining the primary biochemical pathways. Effectors of transcript abundance that could be integrated into such a network of relationships include not only hormones and drugs, as shown here, but also mutations in relevant pathways. For example, to further dissect the multiple effects of TGFβ some obvious targets for fingerprinting include cell lines that carry mutations in the Rb pathway. Another potentially rich source of mutants would result from the disruption of normal expression of genes that are initially identified as being regulated using RAP-PCR. This as yet theoretical iterative process of RAP-PCR, gene identification and disruption, followed by RAP-PCR on the resulting mutant we have termed an 'epistatic walk'.

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