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A gene expression screen

(cDNA subtraction/amphibian metamorphosis/tail resorption/gene expression/thyroid hormone)

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ABSTRACT **A gene expression screen identifies mRNAs that differ in abundance between two mRNA mixtures by a subtractive hybridization method. The two mRNA populations are converted to double-stranded cDNAs, fragmented, and ligated to linkers for polymerase chain reaction (PCR) amplification. The multiple cDNA fragments isolated from any given gene can be treated as alleles in a genetic screen. Probability analysis of the frequency with which multiple alleles are found provides an estimation of the total number of up- and down-regulated genes. We have applied this method to genes that are differentially expressed in amphibian tadpole tail tissue in the first 24 hr after thyroid hormone treatment, which ultimately induces tail resorption. We estimate that there are about 30 up-regulated genes; 16 have been isolated.**

A genetic screen is the traditional method for identifying genes involved in a complex biological process. An estimation of the total number of genes that are involved in a trait is tabulated from the frequency with which different mutations (alleles) in the same gene are identified (1). If a transposable element is used to produce random mutations, then it also tags genes for isolation by cloning methods (2). Thus, a genetic screen can accomplish three essential goals—it estimates the number of genes that are involved in the process, marks them for isolation, and guarantees by the altered phenotype of the mutant that the gene is directly or indirectly involved in the function under study.

A limitation of genetic screens is that they are applicable to just a few "genetic" organisms with short life cycles. There are many complex biological systems, organs, and tissues and physiological states that can only be studied in organisms that are unsuited for genetic screens. There are hormonal and pharmacological agents that affect gene expression but whose action cannot be studied by traditional genetics. The identification and characterization of genes that cause human disease is anticipated to rely not only on traditional genetics but also on methods of biochemistry and molecular biology—namely, the physical mapping and sequencing of the human genome.

Another method that has been used to identify constellations of genes that are involved in some biological event is subtractive hybridization (3–8), which results in the isolation of those mRNAs (in the form of cDNA) whose concentrations differ between two complex mixtures of mRNA. One of the two mRNAs is from the control tissue; the other is isolated from tissue during or after the change being studied. An mRNA that differs in abundance between the two mRNA populations is suspected to be related to the event. Two early uses of subtractive library technology were the identification of genes that are activated at the gastrulation stage of *Xenopus laevis* (8) and cloning of the T-cell receptor (5). Since those original studies, there have been many applications and modifications of subtractive library methodology, more recently incorporating PCR technology (9–13).

This paper describes a subtractive library method that is analogous to a genetic screen in the sense that it can estimate the number of, and therefore lead to the isolation of, virtually all up- and down-regulated genes.

The gene expression screen is applied here to thyroid hormone-induced tadpole tail regression, the final change in amphibian metamorphosis, which occurs at metamorphic "climax" when the endogenous thyroid hormone is at its highest level (14). Tail resorption is genetically programmed and cell-autonomous (15). It can be induced prematurely in premetamorphic tadpoles or in cultured isolated tails by high doses of thyroid hormone (15). After addition of thyroid hormone, morphological changes presumably caused by the induction of various lytic enzymes are first visible after 2–3 days (15). The earliest thyroid hormone response genes that have been identified to date in any tadpole tissue are the two thyroid receptor β genes, which are up-regulated beginning 4–8 hr after addition of thyroid hormone (16).

The object of this gene expression screen was to identify the genes whose expression is up- or down-regulated in tails within the first 24 hr after hormone treatment.

MATERIALS AND METHODS

The enrichment method to isolate up-regulated genes is summarized in Fig. 1.

Preparation of RNA and cDNA. One hundred stage 54 tadpoles (17) were treated with or without 100 nM T_3 (thyroid hormone) for 24 hr in dechlorinated tap water. The tadpoles were precooled in ice water before their tails were amputated. In all succeeding steps the control (−) and T_3-treated (+) RNAs were isolated and treated identically in parallel reactions. Total tail RNA was isolated by the guanidinium/CsCl gradient method (18). Poly(A)$^+$ RNA was selected by passage through an oligo(dT) column (Pharmacia, type 7) (19).

Oligo(dT) was used to prime the first strand of cDNA synthesis from 5 μg of poly(A)$^+$ RNA. Double-stranded cDNA was synthesized using an Invitrogen kit, and it was divided into four aliquots for future use. One portion was used to construct a cDNA library. The average size insert was 2 to 3 kilobases (kb); the largest was 5 kb. Therefore, the largest mRNAs (Table 1) were not represented by full-length cDNAs.

Restriction Enzyme Digestion, Linker Ligation, and PCR Amplification. To prepare cDNA fragments suitable for PCR amplification, two aliquots of double-stranded cDNA were digested completely with *Alu* I and *Alu* I plus *Rsa* I separately and then ligated with 10 μg of a double-stranded phosphorylated oligodeoxynucleotide linker, which had one blunt end and one 4-base 3′ protruding end [CTCTTGCTTGAATTCGGACTA and TAGTCCGAATTCAAGCAAGAGCACA (10)]. The linker had an *Eco*RI site near the flush end. Half of each of the two ligation reactions (*Alu* I-digested and *Alu* I/*Rsa* I-digested) were electrophoresed through a 1.4% low-

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Abbreviation: T_3, 3,3′,5-triiodo-L-thyronine.

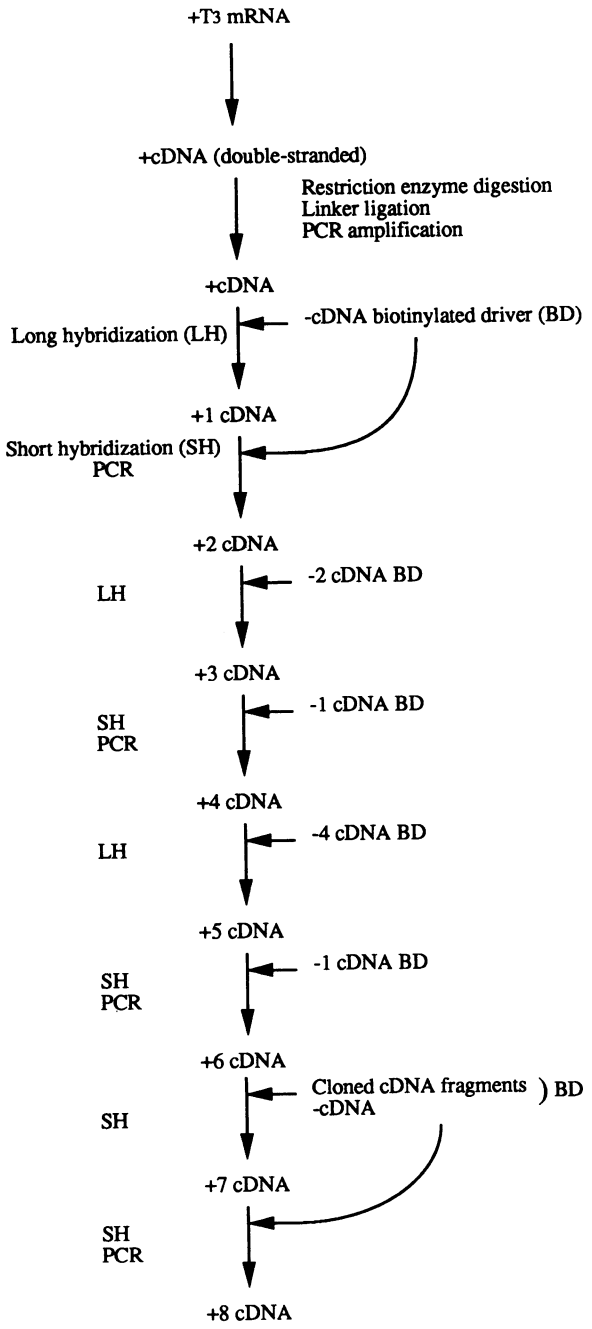

FIG. 1. Flow diagram for isolation of up-regulated genes. A plus sign (+) refers to the mRNA isolated from tadpole tails treated with thyroid hormone (3,3',5-triiodo-L-thyronine, T_3) for 24 hr, as well as the cDNAs derived from this + mRNA; − refers to mRNA and cDNAs from untreated tadpoles. LH, long hybridization; SH, short hybridization; BD, biotinylated driver DNA. The exact opposite protocol is carried out simultaneously to obtain the down-regulated genes.

melting agarose gel for a short distance to remove the unligated linkers. The linker-ligated *Alu* I and *Alu* I/*Rsa* I cDNA fragments in the size range of 0.2–2 kb were combined.

Linker-ligated cDNA fragments in agarose were amplified directly by PCR. One microliter of melted agarose was used for a 100-μl PCR mixture (94°C, 1 min; 50°C, 1 min; 72°C, 2 min, with 25 sec of autoextension per cycle; 30 cycles) and 20 such PCRs were performed for each of the − and + cDNA samples, producing about 350 μg of each cDNA. The amplified − and + cDNA fragments were the starting material for subtractive hybridization.

Table 1. Summary of T_3-induced up- and down-regulated genes

Gene no.	mRNA size, kb	No. of fragments	Fold induction*	mRNA copies per cell†
		Up-regulated genes		
1	8, 10	2	10	40
2	10	1	8	20
3	9	1	10	15
4	8	1	16	15
5	5, 7	3	14	10
6	10	2	6	20
7	6	1	6	120
8	5, 3, 1	1	>20	80
9	5	2	>20	80
10	5	4	>20	120
11	3	1	7	260
12	3	2	>20	40
13	3	1	6	30
14	3	1	6	20
15	1.6	1	10	120
16	1.6	1	10	15
		Down-regulated genes		
17	1.6	1	14	220
18	1.6	2	14	280
19	7	1	5	1000
20	8	1	13	220

*Relative mRNA content in the tail measured by Northern blot before and after 24 hr of thyroid hormone treatment.

†Estimated by comparing Northern and genomic Southern signals when hybridized together. We have assumed that each cell contains 500,000 mRNA molecules of 2.5-kb average size and that the haploid genome size of *X. laevis* is 3×10^9 base pairs. Gene 6 has been identified as thyroid hormone receptor β.

Subtractive Hybridization. One hundred micrograms of PCR-amplified cDNA was digested with 1500 units of *Eco*RI at 37°C for 2 hr to cleave the linker so that residual driver DNA fragments could not be amplified later (10). The driver DNA (100 μg) was mixed with 100 μl of Photoprobe biotin (Vector Laboratories), irradiated with a 270-W sunlamp, and processed as described (9, 10, 20, 21). The photobiotinylation reaction was repeated once to increase the density of biotin molecules so that the biotinylated driver DNA (BD) could be removed more efficiently.

Biotinylated driver (100 μg) and nonbiotinylated tracer (5 μg) DNAs were mixed, precipitated, and redissolved in 20 μl of 10 mM Tris/1 mM EDTA, pH 8. The DNA mixture was boiled for 3 min, briefly centrifuged to collect condensed water, mixed with 20 μl of 2× hybridization buffer (10), overlaid with mineral oil, and boiled again for 3 min to ensure complete denaturation. The denatured cDNA samples were incubated in a 68°C water bath for 20 hr [long hybridization (LH)]. Then enough buffer (10 mM Hepes/1 mM EDTA, pH 7.6) prewarmed at 55°C was added to bring the final NaCl concentration to about 0.1 M, which we found to be suitable to bind streptavidin to biotinylated DNA efficiently. The tubes were then incubated at 55°C for 5 min, and the aqueous phases were transferred into fresh tubes. Twenty microliters of streptavidin (2 μg/μl in 0.15 M NaCl/10 mM Hepes/1 mM EDTA, pH 7.6) was mixed with the hybridized cDNA solution and incubated at room temperature for 20 min to form complexes with biotinylated DNA, and protein and protein–DNA complexes were removed by extraction with an equal volume of $CHCl_3$/phenol (1:1, vol/vol). The streptavidin binding and $CHCl_3$/phenol extraction steps were repeated until there was no visible protein–DNA complex at the interface between the organic and aqueous phases. Usually it took four or five repeated extractions with streptavidin to remove >99% of the biotinylated DNA. An additional two

organic extractions were followed by $CHCl_3$ extraction. The subtracted tracer cDNA (+1 cDNA or −1 cDNA) was mixed with 50 μg of biotinylated *Eco*RI-treated driver DNA, as before, and ethanol-precipitated. The rinsed and dried pellet was resuspended in 40 μl of hybridization buffer and incubated for just 2 hr [short hybridization (SH)]. Biotinylated DNA was removed as before, and the enriched tracer DNA was ethanol-precipitated. The DNA pellet (+2 cDNA or −2 cDNA) was rinsed with ethanol, dried, and resuspended in 100 μl 10 mM Tris/1 mM EDTA, pH 8. Sixty microliters of 2 cDNA was amplified by PCR as before; 3 μl was used in each of 20 PCRs. The product was purified, and 100 μg of the PCR product of 2 cDNA was treated with *Eco*RI and biotinylated for use as driver for the next cycle of subtractive enrichment. Long hybridization used 100 μg of biotinylated 2 cDNA driver and 5 μg of nonbiotinylated 2 cDNA tracer. This was followed by a short hybridization with 50 μg of 1 cDNA driver, producing 4 cDNA (Fig. 1).

An essential aspect of this subtractive enrichment procedure is the use of both long and short subtractive hybridization steps to remove the common DNA fragments (Figs. 1 and 2). Long hybridization is needed to suppress the highly complex rare common cDNAs that comprise 50–60% of the total cDNA mixture. However, long hybridization does not efficiently reduce the abundant common cDNAs and can actually suppress some differentially expressed cDNAs that have a baseline level in the driver cDNA. Thus, the short hybridization is used to suppress the abundant common mRNAs (see actin in Fig. 2). Up-regulated genes were suppressed greatly in −1 cDNA and down-regulated genes were suppressed in +1 cDNA, making these cDNAs better drivers than the initial PCR-amplified cDNAs for short-term hybridization steps.

The enriched + cDNA hybridizes only with itself and not detectably with enriched − cDNA and vice versa (result not shown). Further enrichment can be accomplished by driving out the most abundant enriched cDNA fragments, yielding 7 and 8 cDNA (Fig. 1).

RESULTS

Cloning and Analysis of Up- and Down-Regulated cDNA Fragments. After three cycles of subtractive enrichment, + and − enriched cDNA fragments were amplified with 30 cycles of PCR. The amplified cDNA was purified, and 3 μg was subjected to an additional cycle of PCR amplification (90°C, 5 min; 50°C, 1 min; 72°C, 120 min) to ensure that all cDNA fragments were double-stranded. The products were cleaved with *Eco*RI and ligated to dephosphorylated pBluescript vector (Stratagene) for transformation into competent *Escherichia coli* DH5 cells.

We screened about 3000 colonies from each enriched cDNA library. Approximately one-third of the colonies from a library of +6 cDNA hybridized detectably with probe prepared from the same enriched cDNA. The same was true for −6 cDNA fragments that were cloned and then hybridized with the same −6 cDNA as probe. The colonies that do not show detectable hybridization with the radioactive cDNA are clones of those cDNAs whose abundance is rare in the cDNA. They are derived mainly from the common mRNAs and have been suppressed but not eliminated by the enrichment procedure. The essential goal of any subtractive library procedure is to enrich the probe for the desired cDNA fragments. An individual cDNA fragment must reach a concentration of greater than about 0.2% of the DNA probe before it hybridizes visibly with a colony containing that insert. All of the positive colonies contain inserts from up- or down-regulated mRNAs so that replica plating for comparative hybridization with a control probe is not necessary.

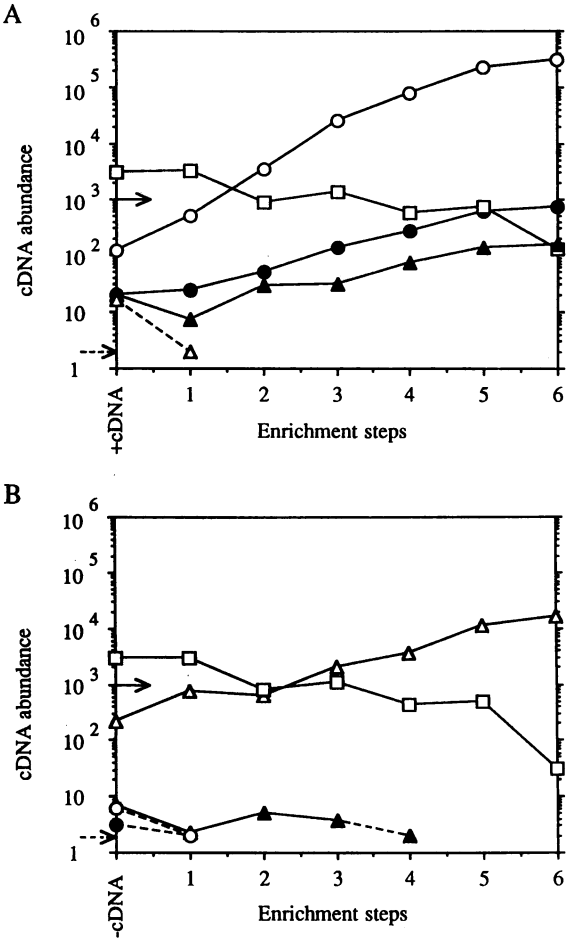

FIG. 2. Enrichment of up-regulated genes (*A*), using + cDNA as tracer and − cDNA as driver; and enrichment of down-regulated genes (*B*), using − cDNA as tracer and + cDNA as driver. The enrichment was assayed by PCR Southern analysis. The flow chart (Fig. 1) and text detail each enrichment step. ●, Up-regulated gene 6 (thyroid hormone receptor β); ○, up-regulated gene 10; □, actin (22); ▲, thyroid hormone receptor α; △, down-regulated gene 17. The cDNA abundance is the number of molecules of each fragment for every 500,000 cDNA molecules at each step of enrichment. Upper arrow, minimum abundance of a specific DNA fragment required (in probes) to generate hybridization signal for colonies that contain the fragment in this screen; lower arrow, detection limit of PCR Southern blot analysis using a cloned probe.

Screening Individual Clones and Assigning cDNA Fragments to mRNAs. After the enriched cDNA plasmid library was screened using the same enriched cDNA as probe, 12 positive clones were picked for small-scale preparation of plasmid DNA ("minipreps"). The inserts were excised with *Eco*RI and purified by agarose gel electrophoresis. The inserts were labeled and used to probe mRNA (Fig. 3) and genomic DNA (Southern blots). The original PCR-amplified cDNA samples (− and + cDNA) can be screened by dot blot or Southern blot if the source of mRNA is rare. The original amplified + and − cDNAs, while not containing a precise representation of the starting mRNAs because of nonrandom amplification by PCR, are expected to have amplified each individual fragment identically in the two cDNA preparations. The method depends upon this assumption. Therefore, a comparison of the abundance of any fragment between the starting amplified + and − cDNAs should represent accurately their relative abundance in the original mRNAs. This was found to be the case for each differentially and equally expressed gene that was tested (Fig. 3).

The first batch of 12 minipreps from colonies derived from the enriched + cDNA library contained just two different inserts. Northern analyses showed that both inserts were from up-regulated genes. The two probes together hybridized to >90% of the positive clones from the enriched cDNA. Similarly, one predominant sequence was identified in the enriched − cDNA library and thus corresponded to a down-regulated gene. About 90% of positive clones in the enriched − cDNA library hybridized with this clone.

The abundant enriched fragments must be driven out of the tracer DNA before the other differentially regulated cDNA fragments can be enriched. This is accomplished by short hybridization of these cDNA fragments (20 μg each) mixed with the original (unsubtracted) PCR-amplified − cDNA (5 μg) as driver to 5 μg of +6 cDNA tracer. This step is repeated once (Fig. 1).

Altogether, 30 non-cross-hybridizing cDNA fragments were isolated, 5 from the − cDNA library and 25 from the + cDNA library. It is suggestive evidence that two cDNA fragments are derived from the same mRNA if they hybridize with mRNAs of the same size on Northern blots. However, a conclusive assignment required probing a full-length cDNA library. Fragments that hybridize to the same λ phage plaques are derived from the same cDNA. This analysis showed that 16 different up-regulated and 4 down-regulated genes had been isolated; more than one fragment was found for 6 up- and 1 down-regulated gene (Table 1). The abundance of a particular mRNA in tail was estimated by comparing the signal by Northern blot of total tail RNA with a genomic Southern blot signal when both Northern and Southern filters were hybridized together with the same probe.

DISCUSSION

A Gene Expression Screen. The advantage of dividing each cDNA into multiple fragments is that each cleaved cDNA fragment is analogous to an allele in a genetic screen. Consideration of the number of alleles for each mRNA in Table 1 provides an estimation of the total number of up-regulated genes by the same sort of probability calculation that enables geneticists to estimate the total number of genes that influence some phenotype from a genetic screen (1). In Table 1, a class of up-regulated genes has been identified whose mRNAs are at least 1.6 kb long, present at 10 copies per cell, and 6-fold up-regulated. We have treated the up-regulated genes identified in Table 1 as a single group and applied a Poisson distribution to the frequency with which multiple alleles were found for any gene. This statistical analysis estimates that there are about 30 total up-regulated genes by approximating the number of as yet undetected genes (14 genes) that have the same features as the 16 already identified (Fig. 4).

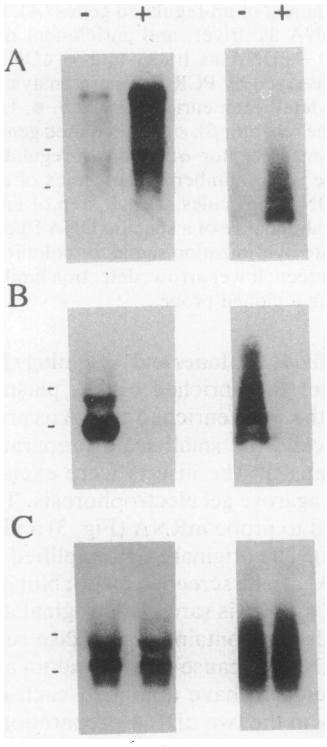

FIG. 3. A comparison of differentially expressed genes in control (−) and thyroid hormone-treated (+) tadpole tail by Northern blot and PCR Southern blot. About 10 μg of total RNA (left two lanes) or 1.5 μg of − or + cDNA (right two lanes) was loaded in each lane. (*A*) Up-regulated gene 6 (thyroid hormone receptor β). (*B*) Down-regulated gene 17. (*C*) *Xenopus* actin (22).

Validity and Limitations of the Gene Expression Screen. The major factor in the method that influences the extent to which any particular cDNA fragment is enriched is the size and sequence of the fragment, since different fragments from the same cDNA can be enriched very differently. If this fact is not to bias the validity of the probability analysis, the following considerations must be true. (*i*) The vast majority of genes must have more than one fragment within the size range that is amplified by PCR. The 25 cDNA fragments were between 0.15 and 0.8 kb long. A computer search of the sequences of 45 randomly selected *Xenopus* cDNAs in the data base revealed that the two restriction enzyme digests (*Alu* I alone and *Alu* I plus *Rsa* I) yield 66 kb out of the total 88 kb of cDNA sequence within the size range for PCR amplification (0.15–0.8 kb). In addition, these restriction enzymes cleave 43 of the 45 cDNAs into multiple fragments within this size range. (*ii*) The alleles (cDNA fragments) from any individual gene are assumed to be amplified randomly by PCR with respect to those from every other gene. In other words, there is no major subset of genes in which all alleles are uniformly resistant to PCR amplification. (*iii*) The same cDNA fragment is amplified identically in the two different mRNAs. The same linker is used for both preparations to

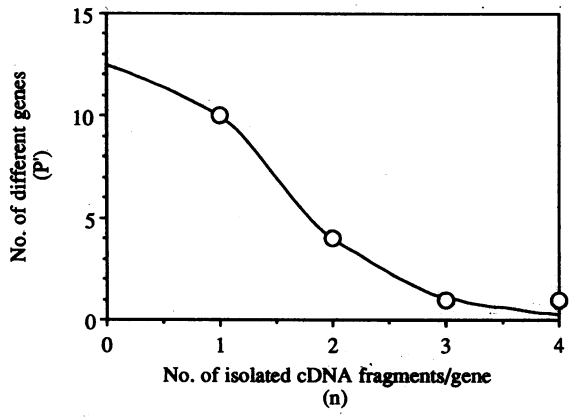

FIG. 4. Poisson distribution analysis of the up-regulated mRNAs identified by this gene expression screen. The line shows the theoretical Poisson distribution curve that best fits the data points, $P'(n) = P(n)N = (e^{-\lambda}\lambda^n/n!)N$, where $\lambda = 0.8$ and n is the number of non-cross-hybridizing fragments isolated for any cDNA. By analogy to a genetic screen n is the number of alleles found for each gene. P is the probability in Poisson distribution, $P'(n)$ is a conversion of P by a constant N, the total number of estimated up-regulated genes, such that $P'(n)$ equals the number of different cDNAs that have n isolated non-cross-hybridizing fragments. Open circles represent the number of isolated cDNAs in the gene expression screen with n isolated non-cross-hybridizing fragments. Where the theoretical Poisson distribution curve meets the vertical axis is an estimation of the number of unidentified up-regulated cDNAs (genes) still present in the library.

ensure this fact. If these assumptions are true, differentially regulated cDNA fragments will be amplified to some extent, inexorably enriched, and in time identified. The frequency with which multiple alleles of the same mRNA are found then is a valid measure of the complexity of all differentially regulated genes.

Individual cDNA fragments were isolated in order of their abundance in the enriched library and then removed to continue the screen. The major factor that influences this abundance is size and sequences of cDNA fragments; otherwise the three features listed in Table 1 (mRNA size, fold induction, and mRNA abundance) would control the abundance of cDNA fragments in enriched samples and the order that each gene is isolated. However, the failure to synthesize full-length cDNA for long mRNAs and the nonrandom amplification overwhelm these three biases so that the genes in Table 1, in effect, comprise a single population. One way of demonstrating this is to repeat the Poisson distribution analysis, omitting either the first 5 or the last 5 cDNA fragments chronologically isolated. The first 5 are the ones that are preferentially enriched to the highest level, while the last 5 are the least abundant cDNAs amongst the 25 that were isolated. Both calculations estimate about 30 total up-regulated genes.

Just as long as PCR can amplify a fragment at all, the process of screening for and then driving out already identified genes, amplifying, and rescreening ultimately raises a fragment's concentration to the point that it can be detected.

The mRNA abundance does not necessarily reflect accurately the amount of the protein that it encodes. Further, this method will not detect important genes critical for a biological event whose mRNA level is unchanged by the event in question. This gene expression screen will not detect differentially regulated genes that lack a poly(A) tail. Very small genes have a greater likelihood of being overlooked because they might not have a site for the restriction enzymes. Random priming of first-strand cDNA synthesis and sonication rather than restriction enzyme digestion to produce the cDNA fragments would include these mRNAs in the gene expression screen. Minimally induced (<6-fold) or low-abundance transcripts (<10 copies per cell) were not found in this screen. However, by adjustment of the driver/tracer ratios (12) and by continued enrichment and removal of more abundant regulated fragments, these genes should also be isolated.

The screen for down-regulated mRNAs has not been extensive enough for a similar calculation.

Applications of a Gene Expression Screen. This method finds differences between two samples of mRNA. It has been pointed out that large genetic deletions can be isolated by gene amplification following subtractive hybridization simply by driving mutant against control genomic DNA (11). Repeated rounds of enrichment and amplification of the remaining tracer genomic DNA, in principal, could isolate ultimately a single-copy gene that was missing in the driver genomic DNA. A deleted 0.5-kb genomic DNA fragment, within the size range that is amplified efficiently by PCR, would need to be enriched about 12,000-fold to comprise 0.2% of total DNA and, therefore, be detected by this method. The highest enrichment in these experiments was about 2000-fold, so one or more additional rounds of subtractive hybridization would be required. This method should identify a single gene responsible for a disease where the amount of mRNA that accumulates in the abnormal cells differs from that in control cells. It should aid in the identification of multiple genes involved in a complex genetic disease where the expression of more than one gene is altered.

A gene expression screen can identify differentially expressed genes related to developmental, physiological, or pharmacological events in any organism. However, the fact that any gene is differentially regulated implies but never proves its functional involvement. A separate functional assay is essential to prove conclusively the importance of a gene in the biological event under study. Nevertheless, the ability to identify all up- and down-regulated genes associated with some complex biological system raises the intriguing possibility that the mere identification of the proteins they encode by sequence homology to known genes in the data base might uncover some unifying principal underlying the biological change.

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