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Induction of human mRNAs by interferon

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We have cloned a set of seven cDNAs corresponding to mRNAs induced by type I interferon in human cells, and are using these probes to characterize the response to interferon in detail. We have studied the kinetics of induction of each mRNA in three different human cell lines, the sensitivity of induction to cycloheximide, the effect of withdrawing interferon and the response of a receptor-positive interferon-resistant cell line.

The synthesis of many mRNAs and proteins is induced when interferons bind to surface receptors of cells. (For a general review, see Lengyel 1982.) Interferon-treated cells are in an antiviral state, and the growth of many different types of RNA and DNA viruses is inhibited. Treatment with interferon can also cause normal or transformed cells to stop growing. The response of cells to interferon is pleiotropic: expression of many different proteins is required to establish the different antiviral pathways and the antigrowth response. Only a handful of interferon-induced proteins have been revealed by the techniques used up to now, and only four or five of these have reasonably well defined functions, primarily in establishing the resistance of interferon-treated cells to RNA viruses. No protein that functions solely in the antigrowth response has yet been identified.

Other major aspects of the interaction of interferon with cells are even less well understood. What is the nature of the signal produced by the binding of interferon to a cell-surface receptor, and how is this signal generated? By what mechanism does the primary signal cause the eventual accumulation of a specific set of mRNAs and proteins? Type I and type II interferons bind to distinct receptors and induce the synthesis of distinct but overlapping sets of proteins. How is such finely tuned differential control of gene expression achieved? We have approached these problems by cloning cDNAs corresponding to mRNAs induced by treatment with interferon, using a differential method which is unbiased by the functions of the proteins.

We began by searching for a human cell line which would give substantial induction of at least one known protein. Bryan Williams (University of Toronto) found that T98G human neuroblastoma cells (De Mayer *et al.* 1982) responded to type I interferons very well, judging from the level of induction of 2′,5′ oligoadenylate synthetase activity, especially after the cells had been maintained at confluence in a low concentration of serum for several days. We found a sharp increase in the activity of this enzyme about 8.5 h after treatment of T98G cells with interferon, and we made a preparation of polyadenylated RNAs from treated and control cells at this time. The interferon-induced mRNAs were converted to flush-ended, double-stranded cDNAs by the use of reverse transcriptase, the Klenow fragment of *Escherichia coli* DNA polymerase and S1 nuclease. Internal cleavage sites for *Eco*R1 were blocked by *Eco*R1

methylase, R1 linkers were added by blunt-end ligation and the cDNAs were cut with *Eco*R1. They were then fractionated according to size on a column of Bio-Rad A50M, and fractions corresponding to the leading edge and top of the cDNA peak were pooled and cloned. We used a lambda phage vector, which has two major advantages over plasmid vectors. First, *in vitro* packaging allows construction of libraries with high efficiency: we obtained more than 10^7 recombinants per microgram of RNA. Second, differential screening procedures, in which only a very small proportion of the labelled probe is complementary to the sequences in a particular clone, give much lower backgrounds with phage than with plasmids. This advantage is crucial if small differences between relatively weak signals are to be recognized reliably. The particular vector chosen, lambda GT10, was designed by Thanh van Huynh and Ronald Davis (Stanford) and kindly provided by them. It is ideally suited to cDNA cloning. The single R1 site in the vector interrupts the CI repressor gene of lambda, so that insertion of any piece of DNA destroys repressor function and renders the resulting phage incapable of lysogeny. The fraction of phage which receives inserts can be assayed readily by scoring the fraction of clear (non-lysogenic) plaques. We kept this fraction below 10% to minimize double insertions. After construction of the library, non-recombinant phage are readily removed by growing the population on a strain of *E. coli* in which lambda can lysogenize with high frequency.

We screened 11000 plaques differentially with cDNA probes prepared from the mRNAs of treated and control cells, using duplicate lifts on to nitrocellulose. From 130 plaques which appeared positive in the initial screen, 24 survived three rounds of plaque purification and rescreening, and these were classified into seven different families on the basis of several independent criteria, such as size of the induced mRNA, extent of induction of the mRNA, hybridization to Southern transfers, cross-hybridization of cDNAs and partial nucleotide sequences. Some of these properties are listed in table 1. All 24 cDNAs were significantly shorter than the corresponding mRNAs, although longer clones have now been obtained from a new library (from Daudi cells) in which only large cDNAs from the leading edge of the A50M peak were used.

TABLE 1. PROPERTIES OF SEVEN INTERFERON-INDUCED mRNAs

name	approximate length bases	maximum fold induction in T98G cells	induction in interferon-resistant Daudi cells
1–8	800	4–20	—
9–27	850	10–40	—
6–16	1000, 2200, 2600, 4200	5–10 (1000 only)	—
6–26	800	3–5	±
10Q	1100	5–20	±
2A	1700	3–5	+
MTII	600	3–10	+

Only one of the mRNAs corresponds to a known protein; its nucleotide sequence shows it to code for human metallothionein II. Another clone, 1–8, hybridizes to a relatively broad band of mRNAs which average about 800 nucleotides in length. These mRNAs are strongly induced by interferon and represent about 0.5% of the mRNA population after induction. They may be transcribed from a multi-gene family, since hybridization of a 1–8 probe to a Southern transfer prepared from human genomic DNA cut with *Eco*R1 reveals a set of specific bands

totalling more than 100 kilobases. The metallothionein II probe and a probe from clone 6–26 also hybridize to multiple bands in Southern transfers, whereas the other four clones seem to correspond to single-copy genes. Transcription of related RNAs of somewhat different length from a set of related genes could account for the heterogeneity observed for 1–8 in the RNA transfers. From a partial nucleotide sequence of 1–8, we find only one long open reading frame which could code for about 25 hydrophobic amino acids, flanked by hydrophilic regions, a structure typical of the insertion regions of membrane proteins. Burrone & Milstein (1983) have noted the appearance of a protein of molecular mass 16000 Da on the surface of interferon-treated cells. Comparison of the sequence of this protein with the one deduced for 1–8 should reveal any relationship between the two.

All seven mRNAs are induced in human T98G neuroblastoma, Daudi lymphoblastoid and HeLa cells in response to α-interferon. The degree of induction for a particular RNA is similar in the different cells, and ranges from 3- to 40-fold. The probes have been used to follow the response to interferon in all three cell lines, with the use of both RNA transfers and slot blots of total RNA for analysis. Most of the experiments reported below were done with T98G cells and α-interferon, with the slot blot technique (Brown *et al.* 1983). With this method, only a few micrograms of total RNA are needed per analysis, and it is straightforward to base quantitative comparisons on both internal and external standards. Duplicate determinations give easily reproducible results. All of the mRNAs are induced by low doses of interferon, well within the physiological range. In some cases reliable induction can be observed with as little as one international unit per millilitre. For most of the mRNAs, induction is extensive within 8 h of treatment with interferon, and increases only slightly when treatment continued for 16 h more. Pretreatment of the cells with the protein synthesis inhibitor cycloheximide does not inhibit induction of any of the seven RNAs. In fact, induction of the mRNA corresponding to clone 10Q is enhanced in cycloheximide-treated cells, reminiscent of the superinduction of interferon itself in cells similarly treated. Metallothionein II is induced by cycloheximide alone, and also in response to heat shock.

A series of experiments was done in which the cells were treated with interferon for 8 h and then transferred to medium without interferon, after washing. Parallel controls were done with untreated cells and with cells maintained in interferon for the duration of the experiment. In every case, there was no further increase in the levels of interferon-induced mRNAs after withdrawal, showing that interferon must be bound to the receptor continuously for the response to continue. Several of the interferon-induced mRNAs were stable and were maintained in the cells for up to two days in the absence of interferon at the levels found at the time of withdrawal.

Nuclear run-off experiments were done with clone 1–8, which corresponds to the most abundant interferon-induced mRNA. Nuclei were prepared from cells pretreated with interferon for either 30 min or 2 h and were incubated with labelled ribonucleoside triphosphates *in vitro*, in parallel with nuclei prepared from control cells. The labelled RNAs were isolated and the fraction complementary to clone 1–8 was determined by hybridization to the cDNA. This fraction increased 15-fold in 30 min, and did not increase much further in 2 h. The nuclear run-off experiment measures the relative number of RNA polymerases initiated on a particular gene that are capable of continuing transcription during the *in vitro* incubation. Although the measurement is somewhat indirect, it has been correlated reasonably well with the rates of transcription *in vivo* in other systems. Thus, we conclude that the rate of transcription is likely to be nearly maximal for the 1–8 gene 30 min after treatment with interferon has been initiated.

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It is likely that comparable results can be obtained for the other mRNAs, but the experiments are more difficult because these RNAs are much less abundant than 1–8. Experiments now in progress reveal significant induction of 1–8 transcription in 5–10 min.

The induction of all seven mRNAs has been followed in wild type Daudi cells and in an interferon-resistant mutant (see table 1). Daudi cells do not grow in the presence of low levels of interferon, and interferon-resistant mutants can be selected on the basis of growth in the presence of interferon. The particular mutant we have studied (Silverman *et al.* 1982) retains the interferon receptor and thus is probably defective at a step following interferon binding. Three of the mRNAs are not induced in the resistant cells (1–8, 9–27 and 6–16), whereas the other four are.

The 6–16 clone hybridizes to an interesting set of mRNAs. The most abundant is 1 kilobase long and is strongly induced by interferon. In T98G cells, there are also three larger species which are not induced. Different cDNA clones corresponding to the 1.0 and 2.2 kilobase species have been obtained and their nucleotide sequences have been compared. The sequences are identical towards the 3′ ends but then diverge abruptly. After a long stretch of non-identity, here is homology again towards the 5′ ends of the RNAs. Since 6–16 probably corresponds to a single-copy gene, on the basis of Southern hybridizations, the results are most consistent either with the possibility that the 2.2 kilobase species is an unspliced precursor of the 1.0, or that the two are alternative splicing products from a common precursor, perhaps one of the larger RNAs. Sequence analysis of genomic DNA and comparison with the sequences of the two cDNAs should make the relationships clear. At the moment, we have the intriguing possibility that treatment with interferon may lead to quantitative differences in splicing pathways from an induced transcript.

Since we only screened 11000 clones from a much larger library, the seven clones we have worked with so far probably represent only a minority of the interferon-induced cDNAs in the library. The differential method used is biased in favour of relatively abundant mRNAs, which appear more frequently and give stronger signals. However, one of the cDNAs (6–16) corresponds to an mRNA that is induced to a level of only 0.02 % of the total. We also recognize that additional clones could probably be obtained by using mRNAs taken earlier or later after treatment with interferon, by using RNAs from different human cell lines, or by using type II rather than type I interferon.

In summary, we have found that induction of the seven RNAs we have studied occurs at low concentrations of interferon and that its continuous presence is required. Induction is rapid, with control probably at the level of transcription. New protein synthesis is not needed. Thus, it is sensible to use the cDNA clones already in hand to obtain genomic clones, and to search regions of these clones near the transcriptional start sites for homologies in nucleotide sequence which may help to define the regions important in interferon-induced transcription. We shall also be able to assay the ability of such putative control regions to confer interferon-induced transcription on other genes, using constructions introduced into cells by transfection.

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