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The messenger RNA sequences in human fibroblast cells induced with poly rI.rC to produce interferon

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ABSTRACT

Induction of human fibroblast cells with poly rI.rC induces interferon mRNA which can be translated into interferon precursor in wheat germ cell free system or in Xenopus oocytes into biologically active interferon.

The extent of gene expression in the poly rI.rC induced cells was compared to that of the uninduced cells by hybridization of the mRNA to complementary DNA.

Homologous template driven hybridization of cDNA revealed the presence of two clearly defined transitions in the total poly A RNA from the induced cells; abundant class and a scarce class comprising approximately 37,000 diverse species of RNA. Heterologous hybridization of the cDNA with total uninduced mRNA showed that the majority of the mRNA sequences are the same in both the induced and uninduced cells.

The results of the hybridization using cDNA prepared to the fraction enriched for interferon mRNA, however, showed that about 4% of the sequences present in the interferon enriched fraction are not present in the uninduced cells. These differences may result from the poly rI.rC induced alterations in gene expression.

INTRODUCTION

Interferons are extracellular glycoproteins induced in eukaryotic cells by viral infection or by the treatment with double stranded nucleic acids (e.g. poly rI.rC), that induce in the adjacent cells the state in which viral replication is blocked (the "antiviral state"). The high specific activity of interferon (10^9units/mg protein)(1,2) makes the interferon system a very suitable model for the study of gene expression.

The induction of human interferon in fibroblast cells by poly rI.rC is a transient phenomenon; the maximal interferon synthesis is reached within a few hours after the poly rI.rC treatment, and sharply decreases thereafter. There is a close correlation between the production of interferon and relative amount of translatable interferon mRNA in the cells (3,4,5). When the induction is

done in the presence of cycloheximide and cells treated with actinomycin D at the time of maximal rate of interferon synthesis, the shut off of interferon synthesis is delayed (superinduction) and the half life of interferon mRNA in the cells is concomitantly increased (6,7). The shut off of interferon production was shown to be due to a control mechanism which involves new RNA synthesis and possibly a protein synthesis that is responsible for the inactivation of interferon mRNA. Our recent results indicate that the poly rI.rC induces in addition to interferon, a number of polypeptides that were detected within a few hours after induction by both the in vitro translation of poly(A) RNA isolated from the induced cells and by the labeling of the cellular proteins (8).

Our intent in this paper is to compare the extent of gene expression occuring in poly rI.rC induced cells to that in the uninduced cells. We will attempt to define the relative complexity, the degree of homology and the extent of specificity in the total cellular poly(A) containing mRNA population after induction with poly rI.rC by the method developed by Bishop et al (9), which involves hybridization of mRNA to its complementary DNA. We have chosen to study the poly(A) mRNA isolated from the cells induced with poly rI.rC in the presence of cycloheximide. These conditions allow us to separate the process of induction from interferon action; in the cycloheximide treated cells the interferon mRNA is transcribed, but practically no interferon is synthesized to exert its effect on the transcription or to induce the antiviral state.

We have examined the total cellular poly (A) RNA population since our primary interest is interferon mRNA and we detect translatable interferon mRNA activity within both cytoplasmic and nuclear poly(A) RNA.

MATERIALS AND METHODS

Induction and mRNA preparation

Human foreskin fibroblast cells were grown in roller bottles and superinduced for 4½ hrs (10). Total cellular RNA was prepared by guanidine-HCl technique (11) and it is a modification of the method described by Cox (12). Polyadenylated RNA was selected by two passages through an oligo(dT) column and ethanol precipitated.

Poly(A) titration with poly(U):

Poly(A) content of the RNA was determined by titration with radioactive

poly(U) as described by Bishop et al (3). A standard curve was contructed by titrating known amounts of poly(A)$_{130}$ (Miles) with ^{3}H poly(U) (6.29 Ci/mmol; New England Nuclear). mRNA concentration was calculated by assuming poly(A) content to be 10% of mRNA by weight.

Synthesis of cDNA and its size analysis:

Avian myeloblastosis virus (AMV) reverse transcriptase was used to synthesize cDNA under conditions which yield a high proportion of full length copies(14-17). ^{3}H-labeled cDNA was analyzed on linear sucrose density gradient as described (14). Electrophoresis of cDNA in 5% polyacrylamide slab gels in 98% formamide was performed as described (18).

Hybridization and Separation of Unhybridized cDNA

^{3}HcDNA (1.5x10^{7} cpm/µg DNA) was mixed with 200 and 4000 fold excess of template mRNA, previously denatured at 100^{o}C for 1 min. Hybridization was performed in a volume of 2.5µl in 0.6M NaCl-0.01M PIPES (pH 6.7) 2mM EDTA, 0.1% SDS, in sealed capillary tubes at 65^{o}C. Reactions were terminated by quick cooling and digested with S1 nuclease as described (19). Samples removed before exposure to mRNA routinely contained 6-8% S1 nuclease-resistant material. Rot values were calculated from the mRNA concentration determined by ^{3}H-poly (U) binding. Unhybridized cDNA from preparative hybridization was separated on HAP column as described (19).

Partial purification of interferon mRNA by sucrose density gradient:

Poly(A) containing RNA from induced cells was layered on a 11.5 ml of 15-30% linear sucrose gradient in 0.05M NaCl, 0.01M EDTA, 0.01M Tris-HCl (pH 7.4) and 0.2% SDS. The gradient was centrifuged for 22 hr at 30,000 rpm in SW41 rotor at 22^{o}C. 0.9 ml fractions were collected and precipitated with ethanol.

Ten oocytes were injected with each fraction (70nl/oocyte) as described previously (10,20). Oocyte homogenates were assayed for interferon by the cytopathic method of Finter (21) as described previously (10,20).

RESULTS

The total poly(A) RNA from the induced cells was used as a template for the *in vitro* synthesis of cDNA by AMV reverse transcriptase. The efficiency of transcription depended on the enzyme RNA ratio; thus 55 units of the enzyme/µg of RNA yielded 350 ng of cDNA. The efficiency of transcription was

not increased by altering the absolute or relative amounts of reverse transcriptase or template. Analysis of this cDNA on alkaline sucrose density gradient and formamide acrylamide gel showed a heterogeneous product with an average chain length of 1000 nucleotides.

The S-1 resistance of this cDNA after heat denaturation was 6-8%, suggesting that significant second strand synthesis occurred during the transcription.

Poly(A) RNA Sequences in Poly rI.rC Induced Human Fibroblast Cells

A large excess of the total poly(A) RNA from the induced cells was reacted to appropriate Rot values with the homologous cDNA. The hybridization kinetics as determined by digestion with single strand specific S-1 nuclease are shown in Fig 1. Specificity of hybridization is shown by the fact that 85% of cDNA is capable of forming S1 nuclease resistant hybrids with template RNA; the cDNA which fails to hybridize may represent cDNA transcripts too small

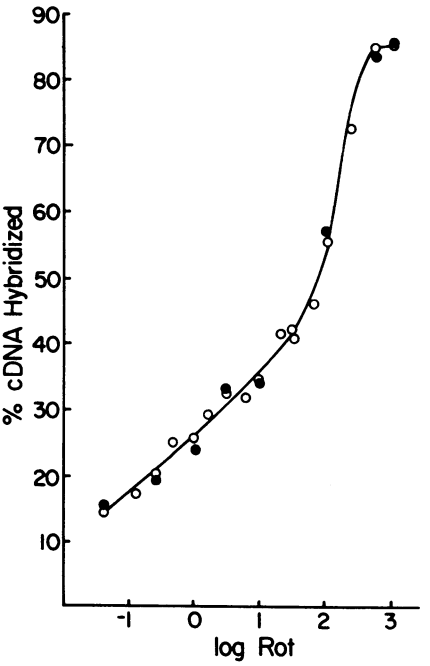

Fig 1. Hybridization of induced cDNA with induced mRNA (o—o), or uninduced mRNA (●—●).
Hybridizations were preformed with 70 pg (1000 cpm) of cDNA per point at a ratio of mRNA to cDNA of 200-4000:1.

to form stable hybrids.

The hybridization kinetics extend over almost five logarithmic decades, revealing a heterogeneous RNA population with diverse distribution of mRNA sequences. To estimate the complexity and abundance level of sequences in each class, we compared this reaction to that described for ovalbumin mRNA-cDNA hybridization, which has been well characterized (22). With the use of this kinetic standard of known base sequence complexity, it is possible to resolve the measured curve into a number of first order components, thus allowing interpretation of the experimental data.

Table 1 compares the RNA frequency class, the kinetic constants, and the base sequence complexity of total mRNAs. The best fit analysis for the poly rI.rC induced poly(A) population indicates the presence of approximately 37,000 different average sized (600,000 daltons) transcript species, separable into two classes. The abundant class represents about 59% and the rare class about 41% of the total poly(A) RNA population. The abundant class contains approximately 260 diverse transcripts of 600,000 daltons. These results suggest that there is a significant difference in the sequence diversity in these two classes and that approximately half of the total poly(A) RNA population in these cells is present in small number of copies.

Specificity of Poly(A) RNA and Sequence Homology Between Induced and Uninduced Cells

By a reciprocal heterologous hybridization it is possible to examine the extent of homology between the uninduced and induced poly(A) RNA population. After plateau has been reached, the difference between homologous and heterologous cDNA hybridization reflects the amount of RNA by mass absent from a

TABLE 1

SEQUENCE COMPLEXITY OF mRNA FROM HUMAN FIBROBLAST CELLS

FINAL % HYBRID	ABUNDANCY CLASS	OBSERVED % cDNA	NORMALIZED % cDNA	OBSERVED Rot ½	Rot ½ IF PURE	NUMBER SEQUENCES *
	Abundant	50	59	1	0.59	260
85	Scarce	35	41	200	82	36,670

Based on calculation with ovalbumin cDNA of 1100 nucleotides long with a Rot ½ of 12.3×10^{-4} moles/litre/sec.

heterologous population.

Fig 1 shows that when the induced cDNA was cross hybridized to uninduced mRNA, the annealing kinetics were similar to that of the homologous hybridization. These results suggest that the sequences in both abundant and rare frequency classes are present in similar frequencies in both induced and uninduced cells and that the expected difference in the rare frequency class is not detectable when cDNA represents the total poly(A) RNA population.

By using fractionated cDNA the differences in common and uncommon sequences between induced and uninduced Poly(A) RNA population can be established more precisely. To facilitate this, the induced cDNA was hybridized to the uninduced poly(A) RNA to a Rot value of 400 to remove most (80%) of the common sequences.The unhybridized cDNA was separated from the hybrids by hydroxylapatite column and used for homologous and heterologous hybridizations. Fig 2 shows that only 20% of this fractionated cDNA, which corresponds to 4% of the total hybridizable cDNA, could be driven into hybrids with the induced and uninduced RNA. Although the terminal hybridization values between the induced and uninduced poly(A) RNA did not differ, the induced poly(A) RNA drove the selected cDNA fraction into hybrids at lower Rot values than the uninduced poly(A) RNA, showing that some of the same sequences present in the induced

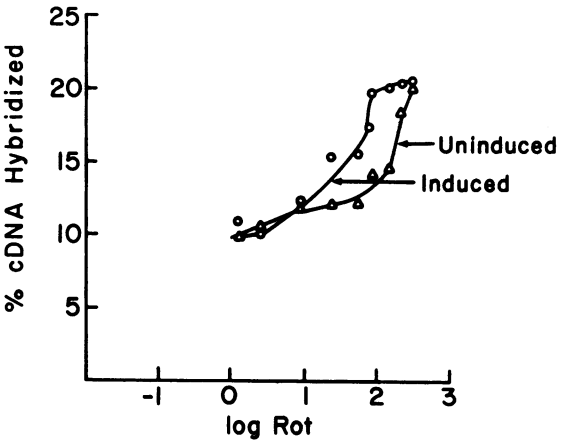

Fig 2. Isolation of rare class cDNA sequences from induced cDNA and its hybridization kinetics with induced and uninduced poly A RNA.

ss cDNA that did not hybridize to the uninduced RNA at a Rot value of 400 was separated and purified as described in Methods and hybridized to induced and uninduced poly A RNA.

cells are also present in uninduced cells, but at a lower frequency. Since the main transition with the induced RNA occurred about 0.5 log units faster than with the uninduced RNA, it appears that these sequences are represented in a 3 fold greater abundance in the induced than in uninduced poly(A) RNA.

Partial purification of interferon mRNA

Reciprocal cross hybridization experiments between the induced and uninduced poly(A) RNA and the fractionated cDNA showed no difference in the plateau of hybridization. These results imply that the sequences specific to poly rI.rC induction (e.g. interferon mRNA) are extremely rare in the total RNA population and therefore undetectable with the transcript cDNA to total cellular poly(A) RNA. To detect the small number of poly(A) RNA sequences which may be specific for interferon induction, interferon mRNA was partially purified from the bulk of cellular poly(A) RNA on a sucrose gradient. The fraction of 12S region containing interferon mRNA (assayed by translation in Xenopus oocytes) was transcribed by reverse transcriptase as described previously. It was estimated by the efficiency of translation in oocytes that the sucrose gradient fraction showed approximately 10 fold enrichment for interferon mRNA. The cDNA prepared for this fraction showed a heterogeneous product with an average chain length of 500 nucleotides. Fig 3 shows the hybridization kinetics are relatively sharper than that obtained with total cDNA (Fig 1), implying that the poly(A) RNA present in this fraction is less heterogeneous. Also shown are the results of heterologous cross hybridization between poly(A) RNA from the uninduced cells and interferon mRNA enriched cDNA. 3-4% of the cDNA hybridizable to the poly(A) RNA from the induced cells failed to react with the poly(A) RNA from uninduced cells. A comparison of the homologous and heterologous curves shows that this 3-4% difference arises at high Rot values and is maintained until the respective plateaus are reached. This would imply that the cDNA specific to the 12S RNA fraction from the induced cells contain non-repetitive sequences which are not represented in the poly(A) RNA population from the uninduced cells.

In an analogous fashion to the comparative studies with cDNA transcribed from the total mRNA, fractionation of the interferon mRNA enriched cDNA was done to characterize more exactly the induced sequences in the size class of interferon mRNA. The sequences common to both induced and uninduced poly(A) RNA were partially removed by hybridization of cDNA to the uninduced poly(A) RNA at Rot of 10 when 45% of cDNA was hybridized. The single stranded cDNA was separated from hybrid on hydroxylapatite column and purified. Fig 4

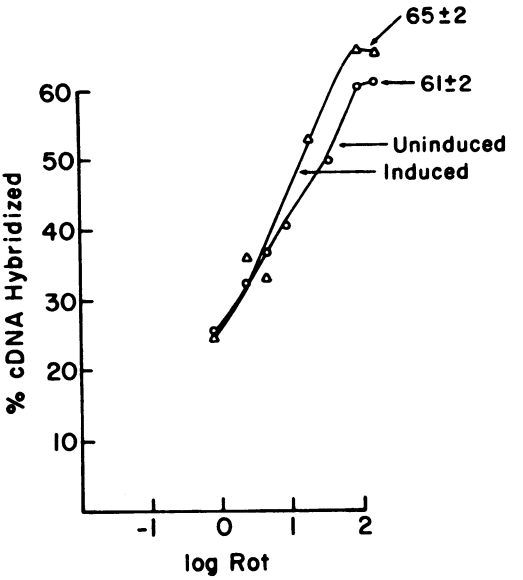

Fig 3. Hybridization of cDNA transcribed from interferon enriched mRNA with induced and uninduced mRNA. Numbers indicate the maximal % of hybridization and represent an average value of two determinations.

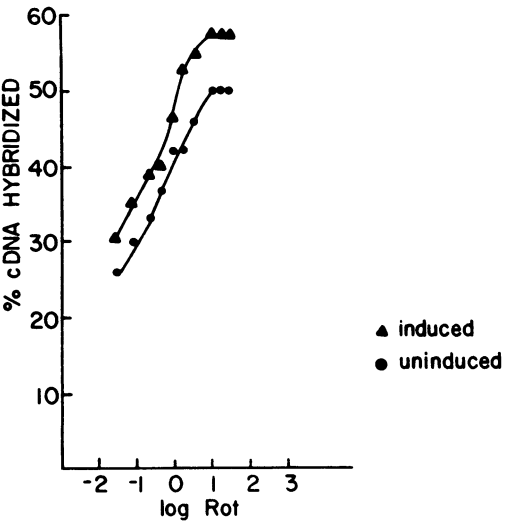

Fig 4. Hybridization of partially purified interferon mRNA enriched cDNA with induced and uninduced mRNA. Interferon mRNA enriched cDNA was hybridized to uninduced mRNA to a Rot of 10 and the single stranded cDNA was separated on a hydroxylapatite and hybridized to induced and uninduced mRNA.

shows the hybridization kinetics of the preselected cDNA fraction with the induced and uninduced poly(A) RNA. At a low Rot value, the poly(A) RNA from induced cells seems to hybridize with a larger proportion of the interferon mRNA enriched selected cDNA fraction than does the uninduced poly(A) RNA. Hybridization with induced mRNA reaches a plateau of 58% compared to 50% for the uninduced poly(A) RNA at similar Rot values. The amplification of the difference in the kinetics of hybridization and plateau (8% vs 4% for the unfractionated interferon mRNA enriched cDNA) indicates that this preselected cDNA fraction has been enriched for unique class sequences.

DISCUSSION

Poly rI.rC induction of human fibroblast cells produces interferon mRNA which is turned over rapidly. When the induction is done in the presence of cycloheximide the interferon mRNA is rendered relatively stable. In order to purify the sequences complementary to interferon mRNA we prepared a cDNA to total poly(A) RNA, which was induced in the presence of cycloheximide. This cDNA was used to evaluate the sequence complexity as well as to estimate the extent of homology between induced and uninduced mRNA population. Furthermore we used an mRNA fraction that was enriched for interferon mRNA by about a 5-10 fold for the preparation of cDNA and then to estimate the proportion of unique sequences between induced and uninduced mRNA.

The usefulness of a cDNA as an analytical probe for hybridization depends on whether the cDNA is fully representative of the RNA from which it is transcribed. The fibroblast cDNA employed in these studies is probably fully representative of fibroblast poly(A) RNA for several reasons. First, the yield of cDNA product to RNA template is high and compares favorably with that reported by Kacian and Myers (15) for poliovirus genome. RNA molecules which are copied are probably copied along most if not all of their chain length, because the size of the cDNA product correlates favorably with that of poly(A) RNA template. The previously observed failure of reverse transcriptase to copy some of the poly(A) RNA molecules in a population is probably a random, but as yet unexplained phenomenon of *in vitro* synthesis (15).

There appears to be a vast difference in the total diversity of transcripts between two abundance classes. The rare copy transcript class contains the vast majority of the total sequence diversity. This compares well with the complexity found in Hela cell mRNA. While the diversity in the abundant class of transcripts compared well with the complexity found in other cell lines,

the diversity among rare transcript class is higher than reported in these cell lines (19,23). It is unlikely that such a large amount of sequence diversity could code for proteins and it is very likely that most of this diversity will be found to be nuclear limited.

The major finding in this report is the large degree of homology between the message species sequences found in fibroblast cells uninduced and induced with poly rI.rC in the presence of cycloheximide. No apparent major differences could be detected in induced mRNA population from that of uninduced mRNA population. The experiments designed to reveal and amplify the difference in two mRNA populations suggested that a small portion of message sequences (about 4%) are present in a 3-fold greater abundance in induced mRNA than in uninduced mRNA.

Several biological differences between mRNA from poly rI.rC induced and uninduced fibroblasts have been established. One is the synthesis of interferon mRNA in the induced cells that can be assayed by translation in Xenopus oocytes or cell free system (4,5,10,20,24,25). Second is the appearance of several mRNA that code for the synthesis of 14,000-70,000 dalton proteins in wheat germ cell free system (8). The proportion of the interferon mRNA in 12S fraction of total mRNA was estimated to be less than 1% (26), which corresponds to less than 0.1% of total mRNA. It is, therefore, not surprising that we fail to detect any differences between the hybridization of cDNA transcribed from the total mRNA from the induced cells with the homologous and heterologous (uninduced) mRNA. This data further indicates that poly rI. rC treatment of human fibroblast does not induce major differences in the transcription of cellular mRNA. When a sequence heterogeneity between induced and uninduced mRNA was analyzed with a probe enriched for interferon mRNA sequences it was found that the poly(A) RNA from induced cells contained 3-4% of unique sequences which are not present in poly(A) RNA population from the uninduced cells. This corresponds to about 0.3-0.4% of cDNA transcribed from the total poly(A) RNA. If these differences are concentrated in the high complexity class this would represent approximately 260 sequences specific for the poly rI.rC induction.

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