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Epidermal growth factor and oncogenes induce transcription of the same cellular mRNA in rat fibroblasts

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We have isolated and sequenced a cloned cDNA corresponding to an mRNA present in significantly higher levels in rat cells transformed by polyoma virus, Rous sarcoma virus, and the cellular oncogene H-*ras* than in the normal parental cell lines. The mRNA transcript is also rapidly induced by the polypeptide growth factor epidermal growth factor, providing a new link between oncogenes and growth factors. Both the growth factor and the oncogenes control expression of the corresponding gene at the transcriptional level. Our results point to the existence of intracellular mechanisms that are common to the action of both growth factors and oncogenes.

Key words: oncogene/growth factor/transcription/*ras*/*src*

Introduction

The recent discoveries that the cellular homologues of the oncogenes v-*sis* and *erbB* are genes coding respectively for platelet derived growth factor (PDGF, Waterfield *et al.*, 1983; Doolittle *et al.*, 1983) and the epidermal growth factor (EGF) receptor (Downward *et al.*, 1984) has firmly established a link between oncogenes and growth factors (Heldin and Westermark, 1984). However, relatively little is known about the mechanisms used by either oncogenes or growth factors to exert their influence on cellular proliferation. Much effort has been concentrated on determining the cellular location of oncogene proteins and in the characterization of the enzymatic activities of oncogene proteins and growth factor receptors (reviewed by Hunter, 1984). Only a few mRNAs have been described whose levels are increased in cells stimulated by growth factors (Courtney *et al.*, 1982; Matrisian *et al.*, 1985; Muller *et al.*, 1984; Linzer and Nathans, 1984; Cochran *et al.*, 1983) or expressing oncogenes (Scott *et al.*, 1983; Brickell *et al.*, 1983; Courtney *et al.*, 1982). The identification of such mRNAs and their protein products, and the characterization of the means used to control their levels, is important as a way of furthering understanding of the mechanisms used by cells to control growth, and may shed light on how these mechanisms are by-passed in the cancerous state. With these aims in mind we have used a cDNA probe enriched for sequences specific for rat fibroblasts transformed by polyoma virus to screen a cDNA library constructed using the phage λgt10. We have isolated a cloned cDNA corresponding to an mRNA present in significantly higher levels in rat cells transformed by polyoma virus, Rous sarcoma virus (RSV) or the cellular oncogene H-*ras* relative to the normal parental cell lines. This mRNA, referred to as pTR1 RNA, is also specifically induced by the polypeptide growth factor EGF. We have shown that control of pTR1 RNA levels takes place at the transcriptional level, suggesting that this system will prove particularly useful for investigating the possibly interlocking mechanisms of action of oncogenes and growth factors.

Results

Isolation of a transformation-induced cDNA

A cDNA library was made from poly(A)$^+$ RNA isolated from PyT21 cells [polyoma virus-transformed rat FR3T3 fibroblasts (Rassoulzadegan *et al.*, 1982)] using the λgt10 system (Huynh *et al.*, 1985). Double-stranded cDNA carrying *Eco*RI linkers was cloned into the unique *Eco*RI site of λgt10 DNA to generate a recombinant phage library. The library was screened with an enriched probe prepared by hybridization-subtraction as described by Hedrick *et al.* (1984). [^{32}P]cDNA reverse transcribed from PyT21 mRNA was hybridized to an 8-fold excess of FR3T3 mRNA, and double-stranded material removed by chromatography on hydroxylapatite. The single-stranded fraction was subjected to a second cycle of hybridization and chromatography to generate a cDNA probe coresponding to ~0.5% of the starting cDNA, taking a 50% yield per cycle into account. This probe, enriched for sequences present at a higher level in PyT21 cells relative to FR3T3 cells, was used to screen ~20 000 recombinant phage. Eight such phage gave a positive signal with the enriched probe, and only a very weak signal with a probe prepared form FR3T3 cell mRNA (data not shown). Cross-hybridization experiments and restriction enzyme mapping suggested that the cDNA inserts of four of these phages corresponded to the same mRNA. The largest of these cDNA inserts was 1.6 kb. For convenience, this fragment was subcloned into the *Eco*RI site of pUN121 (Nilsson *et al.*, 1983) to generate pTR1. This material was used to investigate the expression of the corresponding gene in a variety of transformed cell lines.

Several oncogenes induce expression of the pTR1 RNA

We examined cell lines derived from both FR3T3 and Rat-1 fibroblasts by transformation with polyoma virus (either the entire virus or only the middle T oncogene), RSV or the human cellular oncogene H-*ras*. Total cytoplasmic RNA from these lines was size-fractionated by electrophoresis, transferred to nitrocellulose filters, and hybridized with the nick-translated pTR1 probe. In each of the transformed lines the labeled plasmid hybridizes to an ~1.9-kb transcript (abbreviated henceforth to pTR1 RNA). No signal is detected, however, in the normal parental cell lines (Figure 1a,b). This suggests that expression of various oncogenes results in a marked elevation in the level of pTR1 RNA. To determine whether the induction of the pTR1 RNA is indeed a result of oncogene expression, we also examined a Rat-1 cell line transformed by a temperature-sensitive variant of RSV [LA-24 (Magun *et al.*, 1979), Figure 1c]. The pTR1 RNA is clearly visible at the permissive temperature (35°C), and is undetectable with the same exposure time when the cells are grown at non-permissive temperatures (39°C).

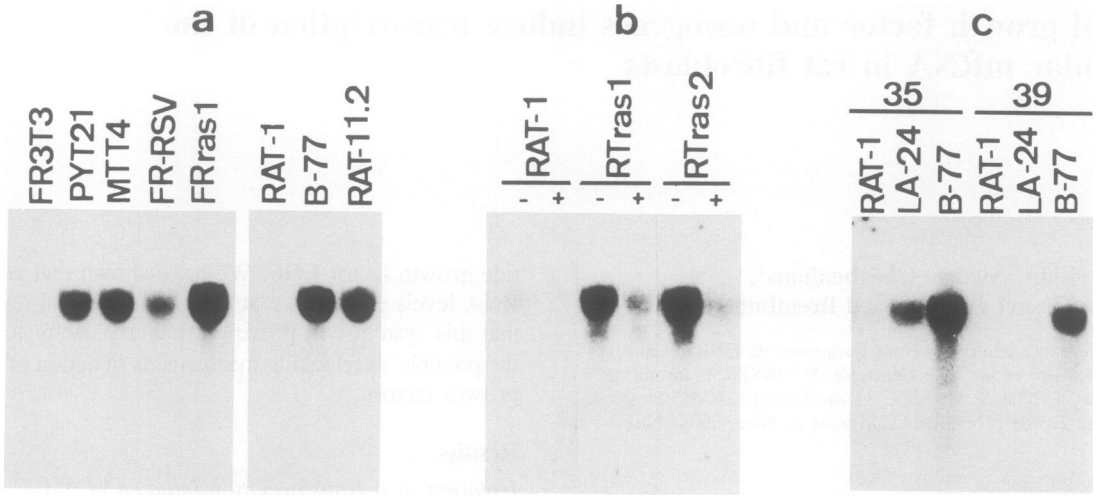


Fig. 1. Transfer hybridization analysis of pTR1 RNA transcripts in normal and transformed cells. Each lane represents 10 μg of total cytoplasmic RNA. (**a**) Tissue culture cells were maintained in 10% fetal calf serum. Similar filters were hybridized with a cloned cDNA selected at random from the original library (pF) and all samples exhibited roughly equivalent levels of pF-hybridizing RNA. In addition, the FR3T3, PyT21 and MTT4 samples contained equivalent amounts of RNA hybridizing to another random cDNA clone and to the cDNAs for four glycolytic enzymes (Matrisian *et al.* 1985). (**b**) Cells were either grown and collected in the presence of 10% fetal calf serum (+), or were washed twice with PBS and exposed to serum-free medium for 24 h before RNA isolation (−). Hybridization of a similar filter with pF demonstrated equivalent levels of pF hybridizing RNA in all lanes. (**c**) LA-24 cells were plated overnight at 39°C, and then half of the flasks were transferred to 35°C. The cells were allowed to grow for 2 days. At that time half of the flasks were collected (in serum, results of the experiment are not shown but differ from those in Figure **c** only quantitatively). The remaining flasks were washed twice with PBS and the medium replaced with serum-free medium. Cells were harvested 24 h later. Identical filters hybridized with the control clones pF and pD5 (Matrisian *et al.*, 1985) demonstrated equivalent levels of hybridizing RNA in all lanes (data not shown).

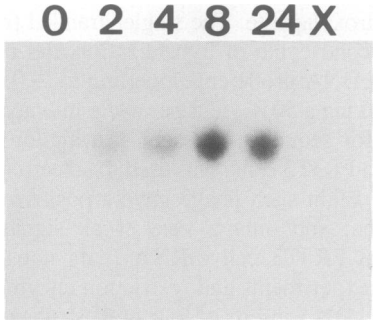


Fig. 2. EGF-stimulation of pTR1 RNA. Rat-1 cells were serum-starved for 2 days and 20 ng/ml of culture grade (P-10 purified) EGF (Savage and Cohen, 1972) was added at $t = 0$. The cells were harvested at the indicated times (in hours) and Northern blots were prepared as described previously. X = RNA from cells growing exponentially in 10% fetal calf serum. Similar filters hybridized with pF and pD5 showed equivalent levels of hybridizing RNA in each lane.

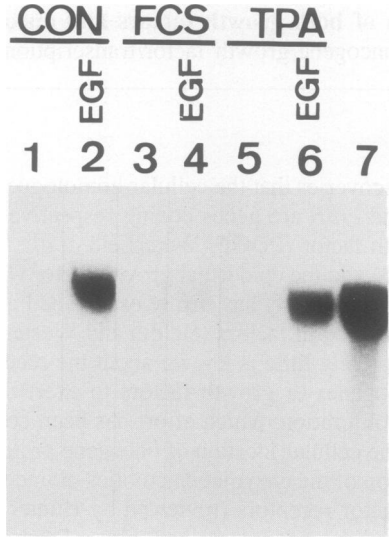
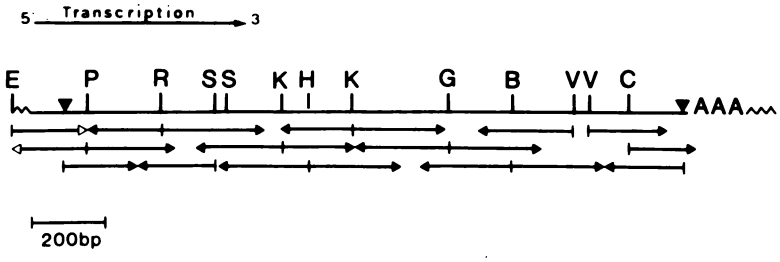


Fig. 3. Effect of serum and phorbol ester tumor promoter on pTR1 RNA levels. Rat-1 cells were serum-starved for 2 days. The flasks represented by **lanes 5** and **6** were treated at $t = -1$ h with the phorbol ester TPA (Sigma, Inc.) at a final concentration of 100 ng/ml and 0.1% DMSO. At $t = 0$, 2 ml of the serum-free medium was removed from the 20 ml in flasks represented in **lanes 3** and **4**, and 2 ml of fetal calf serum was added to obtain 10% serum final concentration. Culture grade EGF was then added to flasks represented in **lanes 2, 4** and **6** at a final concentration of 20 ng/ml. RNA was harvested from all cells at $t = 24$ h and Northern blots prepared as described. **Lane 7** represents RNA isolated from Rat-1 1.2 cells (polyoma middle T-transformed Rat-1 cells). Hybridization of similar filters with pF and pD5 demonstrated equivalent levels of hybridizing RNA in all lanes.

EGF induces expression of the pTR1 RNA

Because of the current interest in the hypothesis that the transformed phenotype is mediated in part by secreted factors [i.e., transforming growth factors, TGFs (Roberts *et al.*, 1983)], we examined medium conditioned by PyT21 cells to determine if such a factor could be responsible for the induction of pTR1 RNA. The medium (serum-free, conditioned by PyT21 or FR3T3 cells for 24 h) was applied to FR3T3 cells in the presence or absence of 5 ng/ml EGF, as EGF is known to potentiate the transforming effects of TGF-β (Roberts *et al.*, 1983). Although no significant difference was observed in the hybridization signal of cells treated with conditioned medium from FR3T3 or PyT21 cells, all cells treated with EGF expressed a considerable amount of pTR1 RNA (data not shown). We therefore further investigated the effects of EGF on pTR1 RNA levels.

Rat-1 cells were grown to confluence, serum-starved for 2 days, and then exposed to 20 ng/ml of culture grade EGF. Total cytoplasmic RNA was harvested at the indicated times and Northern blots were prepared as described above. EGF-stimulated cells demonstrated a steady increase in pTR1 RNA levels that was detectable at 2 h and increased up to 8 h after EGF addition

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                                                                                                                    5'..AAGGAGGCAGCAAAGAACCCGCTGAGAGCAGTGCAGAACTGTGGGAAGCCAGTGGAA

58                                          88                                          118
 ATG AAA GGG CTC CCA GTC CTG CTG TGG CTG TGT ACG GCT GTG TGC TCA TCC TAC CCA TTG CAT GGC AGT GAA GAA GAT GCT GGC ATG GAG
 Met Lys Gly Leu Pro Val Leu Leu Trp Leu Cys Thr Ala Val Cys Ser Ser Tyr Pro Leu His Gly Ser Glu Glu Asp Ala Gly Met Glu

148                                         178                                         208
 GTT CTG CAG AAA TAC CTA GAA AAC TAC TAT GGT CTT GAA AAG GAT GTG AAG CAG TTT ACT AAG AAA AAG GAC AGT AGC CCT GTT GTC AAA
 Val Leu Gln Lys Tyr Leu Glu Asn Tyr Tyr Gly Leu Glu Lys Asp Val Lys Gln Phe Thr Lys Lys Lys Asp Ser Ser Pro Val Val Lys

238                                         268                                         298
 AAA ATT CAA GAA ATG CAG AAG TTC CTT GGG CTG AAG ATG ACA GGG AAG CTG GAC TCG AAC ACT ATG GAG CTG ATG CAC AAG CCC CGG TGT
 Lys Ile Gln Glu Met Gln Lys Phe Leu Gly Leu Lys Met Thr Gly Lys Leu Asp Ser Asn Thr Met Glu Leu Met His Lys Pro Arg Cys

328                                         358                                         388
 GGT GTT CCC GAC GTC GGT GGC TTC AGT ACC TTT CCA GGT TCA CCC AAA TGG AGG AAA AAC CAC ATC TCC TAC AGG ATT GTG AAT TAT ACA
 Gly Val Pro Asp Val Gly Gly Phe Ser Thr Phe Pro Gly Ser Pro Lys Trp Arg Lys Asn His Ile Ser Tyr Arg Ile Val Asn Tyr Thr

418                                         448                                         478
 CTG GAT TTA CCA AGA GAG AGT GTG GAT TCT GCC ATT GAG AGA GCT TTG AAG GTC TGG GAG GAG GTG ACC CCA CTC ACA TTC TCC AGG ATC
 Leu Asp Leu Pro Arg Glu Ser Val Asp Ser Ala Ile Glu Arg Ala Leu Lys Val Trp Glu Glu Val Thr Pro Leu Thr Phe Ser Arg Ile

508                                         538                                         568
 TCT GAA GGA GAG GCT GAC ATA ATG ATC TCC TTT GCA GTT GAA GAA CAT GGA GAC TTT ATC CCT TTT GAT GGG CCT GGA ATG GTC TTG GCT
 Ser Glu Gly Glu Ala Asp Ile Met Ile Ser Phe Ala Val Glu Glu His Gly Asp Phe Ile Pro Phe Asp Gly Pro Gly Met Val Leu Ala

598                                         628                                         658
 CAT GCC TAT GCC CCT GGA CCA GGG ACC AAT GGA GAT GCT CAC TTT GAT GAT GAT GAA CGA TGG ACA GAT GAT GTC ACA GGT ACC AAC CTA
 His Ala Tyr Ala Pro Gly Pro Gly Thr Asn Gly Asp Ala His Phe Asp Asp Asp Glu Arg Trp Thr Asp Asp Val Thr Gly Thr Asn Leu

688                                         718                                         748
 TTC CTG GTT GCT GCT CAT GAA CTT GGC CAC TCC CTG GGT CTC TTT CAC TCA GCC AAT GCT GAA GCT TTG ATG TAC CCA GTC TAC AAG TCC
 Phe Leu Val Ala Ala His Glu Leu Gly His Ser Leu Gly Leu Phe His Ser Ala Asn Ala Glu Ala Leu Met Tyr Pro Val Tyr Lys Ser

778                                         808                                         838
 TCC ACA GAC CTG GCC CGT TTC CAT CTC TCT CAA GAT GAT GTA GAT GGT ATT CAA TCC CTC TAT GGA CCT CCC ACA GAA TCC CCT GAT GTC
 Ser Thr Asp Leu Ala Arg Phe His Leu Ser Gln Asp Asp Val Asp Gly Ile Gln Ser Leu Tyr Gly Pro Pro Thr Glu Ser Pro Asp Val

868                                         898                                         928
 CTC GTG GTA CCC ACC AAA TCT AAC TCT CTG GAC CCT GAG ACC TTA CCA ATG TGT AGC TCT GCT TTG TCC TTC GAT GCA GTC AGC ACC CTG
 Leu Val Val Pro Thr Lys Ser Asn Ser Leu Asp Pro Glu Thr Leu Pro Met Cys Ser Ser Ala Leu Ser Phe Asp Ala Val Ser Thr Leu

958                                         988                                         1018
 CGG GGA GAA GTC TTG TTC TTT AAA GAC AGG CAC TTT TGG CGA AAA TCT CTC AGG ACC CCT GAG CCT GGC TTT TAT TTG ATC TCT TCA TTT
 Arg Gly Glu Val Leu Phe Phe Lys Asp Arg His Phe Trp Arg Lys Ser Leu Arg Thr Pro Glu Pro Gly Phe Tyr Leu Ile Ser Ser Phe

1048                                        1078                                        1108
 TGG CCG TCT CTT CCA TCC AAC ATG GAT GCT GCA TAT GAA GTT ACT AAC AGA GAC ACT GTT TTC ATT CTT AAA GGA AAT CAG ATC TGG GCT
 Trp Pro Ser Leu Pro Ser Asn Met Asp Ala Ala Tyr Glu Val Thr Asn Arg Asp Thr Val Phe Ile Leu Lys Gly Asn Gln Ile Trp Ala

1138                                        1168                                        1198
 ATC CGA GGT CAT GAA GAG CTA GCA GGT TAT CCT AAA AGC ATT CAC ACT CTG GGC CTC CCT GAA ACC GTC CAG AAG ATC GAT GCA GCC ATT
 Ile Arg Gly His Glu Glu Leu Ala Gly Tyr Pro Lys Ser Ile His Thr Leu Gly Leu Pro Glu Thr Val Gln Lys Ile Asp Ala Ala Ile

1228                                        1258                                        1288
 TCT TTA AAG GAC CAA AAG AAG ACG TAC TTC TTT GTA GAG GAC AAA TTC TGG AGA TTT GAT GAG AAG AAA CAA TCC ATG GAT CCA GAA TTT
 Ser Leu Lys Asp Gln Lys Lys Thr Tyr Phe Phe Val Glu Asp Lys Phe Trp Arg Phe Asp Glu Lys Lys Gln Ser Met Asp Pro Glu Phe

1318                                        1348                                        1378
 CCC AGG AAA ATA GCT GAG AAC TTT CCA GGC ATT GGC ACA AAG GTG GAT GCT GTC TTT GAA GCA TTT GGG TTT CTT TAC TTC TTC AGC GGA
 Pro Arg Lys Ile Ala Glu Asn Phe Pro Gly Ile Gly Thr Lys Val Asp Ala Val Phe Glu Ala Phe Gly Phe Leu Tyr Phe Phe Ser Gly

1408                                        1438                                        1468
 TCT TCA CAG TTG GAG TTT GAT CCA AAT GCA GGG AAA GTG ACC CAC ATA TTG AAG AGC AAC AGC TGG TTT AAT TGT TAA GAA GAT CCA TGG
 Ser Ser Gln Leu Glu Phe Asp Pro Asn Ala Gly Lys Val Thr His Ile Leu Lys Ser Asn Ser Trp Phe Asn Cys ***

1498                        1528                        1558                        1588
 AAGGCGTCGTGTGTTTCAGCTGACCCTGATAGCTCTTCCTCTGAAACTTGGCGCACTGAAGTGGTTTCCTTACTCTAGCATGTGCTATGGCAGAGCAAAATGGGAGCTACATATGGCACC

1618                        1648                        1678                        1708
 AGTCAACCTCAAGTTGTCGAAGGACATTCAGAAGCACTGCTTGTCTTATACTGTGTCAAAGGGAGAGGAGAAAACACACTCCTGGGCTACAGACAAGTAACTGTCTCTGTGTAGATGGTT

1738                        1768
 TGTTTTATTTAATAAAATGGTGTGTCATTTATTTAAAAAAA..3'
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Fig. 4. Nucleotide sequence of pTR1 and deduced protein sequence. **Top**: restriction map of a 'full-length' cDNA clone and sequencing strategy. The limits of the pTRl sequence are shown by vertical arrowheads above the map which represent the *Eco*RI linkers. The rest of the sequence was obtained from the full-length cDNA clone isolated from the 'Okayama and Berg' library (see text). The wavy line represents vector DNA and the solid line represents insert DNA. Filled-in arrows indicate the direction and extent of sequencing using the M13-dideoxy technique (Matrisian *et al.*, 1985) with ^{35}S-labeled (Biggin *et al.*, 1983) dATP and a gradient sequencing gel (Biggin *et al.*, 1983). Open arrows indicate sequence obtained using the Maxam and Gilbert technique (Maxam and Gilbert, 1980). AAA represents the poly(A) tail. E = *Eco*RI, P = *Pst*I, R = *Rsa*I, S = *Sau*3AI, K = *Kpn*I, H = *Hind*III, G = *Bgl*II, V = *Pvu*II, C = *Hinc*II. Only the *Rsa*I and *Sau*3AI sites used for sequencing are shown. **Bottom**: nucleotide sequence of a 'full-length' pTR1 cDNA. The deduced protein sequence is shown. The sequence was determined on both strands apart from nucleotides 520 – 590 and 1382 – 1450.

(Figure 2). By 24 h, the level of pTR1 RNA had decreased slightly.

Because serum contains many growth factors, including EGF, we were puzzled by the induction of pTR1 RNA by EGF but not by serum (Figure 1a). We therefore examined the effects of serum on the EGF response of cells. EGF (20 ng/ml) was added to serum-starved Rat-1 cells in the presence or absence of 10% fetal calf serum. Cells were harvested at 24 h and analyz-

ed for pTR1 RNA. The results shown in Figure 3 (lanes 2 and 4) demonstrate marked reduction in the ability of EGF to increase the level of pTR1 RNA in the presence of serum. These data suggest that there is a factor(s) in serum which can repress the effect of EGF on pTR1 RNA levels.

In the course of the above experiments, we noted that pTR1 RNA, undetectable in Rat-1 cells maintained in serum, could be detected at a low level in cells maintained in serum-free medium (Figure 3, lane 1). A low level of pTR1 RNA is also visible in Rat-1 and LA-24 cells at 39°C in serum-free medium with a longer exposure time of the autoradiogram reproduced in Figure 1c. Furthermore, the *ras*-transformed Rat-1 lines (RTras1 and RTras2) exhibit marked reduction in the levels of pTR1 RNA in the presence of serum (Figure 1b). Serum therefore appears to contain a factor or factors capable of repressing 'endogenous' pTR1 RNA levels as well as pTR1 RNA induced by EGF and possibly also by oncogenes.

As the phorbol ester tumor promoter TPA has been reported to have a number of effects in common with EGF (but also to inhibit the binding of EGF to cellular receptors) (Lee and Weinstein, 1978; Magun *et al.*, 1980) we examined its influence on pTRl RNA levels. However, TPA had no effect on either pTRl RNA levels or on the EGF stimulation of pTRl RNA levels (Figure 3, lanes 5 and 6).

pTR1 RNA has coding potential for a protein of ~53 K

We attempted to identify the pTR1 RNA by sequencing the cDNA insert of pTR1 and regions of a 'full-length' pTR1 cDNA and the corresponding genomic clone. The full-length cDNA was obtained by screening a cDNA library made using the Okayama and Berg (1982) technique. The cDNA obtained is probably complete as a comparison of its sequence (Figure 4) with that of the corresponding genomic clone (Figure 5) shows that the first nucleotide of the cDNA is appropriately positioned relative to the classic TATA or CAAT elements (Breathnach and Chambon, 1981) of eukaryotic promoters. We compared the nucleotide sequence of pTR1 and deduced protein sequence with that of known RNAs or proteins. The putative protein produced by this RNA contains 475 amino acids (mol. wt. ~53 000). A comparison of the sequences with sequences in the European Molecular Biology Laboratory (EMBL) databank or the National Biomedical Research Foundation (NBRF, 'Dayhoff') database revealed no extensive homology with any of the sequences reported therein. In particular the pTR1 sequence shows no homology to the sequence of the major histocompatibility complex (MHC) antigen that is induced by transformation of mouse cells with various viruses and chemical carcinogens (Scott *et al.*, 1983; Brickell *et al.*, 1983). Furthermore, in contrast to both the MHC-like (Scott *et al.*, 1983; Brickell *et al.*, 1983) and VL30 RNA (Courtney *et al.*, 1982), pTR1 does not contain repeated sequences and is encoded by a single-copy gene in the rat genome (our unpublished observations). No homology was observed with the p53 transformation-associated protein (Bienz *et al.*, 1984). (The mol. wt. of the p53 protein deduced from the nucleotide sequence is 43 500.)

5′..AAATGGTCCCATTTGGATGGAAGCAATTATGAGTCAGTTTGCGGGTGACTCTGCAAA

1

TACTGCCACTCTATAAAAGTTGGGCTCAGAAAGGTGGACCTCGAGGAGGCAGCAAAGAA..3′

Fig. 5. Partial sequence of genomic pTR1 DNA (see Materials and methods). The TAT and CAAT boxes are underlined. Sequences found in the pTR1 cDNA clone are in small letters, and the number 1 represents the first nucleotide of the cDNA sequence, shown in Figure 4.

Transcriptional control of pTR1 RNA levels by oncogenes and EGF

To determine whether control of pTR1 levels occurred transcriptionally or post-transcriptionally, nuclei from Rat-1 cells, Rat-1 cells treated with EGF, and Rat-1 cells transformed by polyoma virus middle T protein (line Rat1-1.2) or RSV (line B-77) were used for nuclear run-on experiments in the presence of [^{32}P]CTP. Labelled RNA thus obtained was hybridized to the pTR1 cDNA insert and other control DNA fragments covalently bound to DBM paper. As shown in Figure 6, no pTR1 RNA is detected in nuclei from Rat-1 cells under these conditions. Significant amounts of pTR1 RNA are observed, however, in nuclei from both the transformed cell lines Rat1-1.2 and B-77, and in nuclei from Rat-1 cells exposed to EGF for 2 h. With all samples the

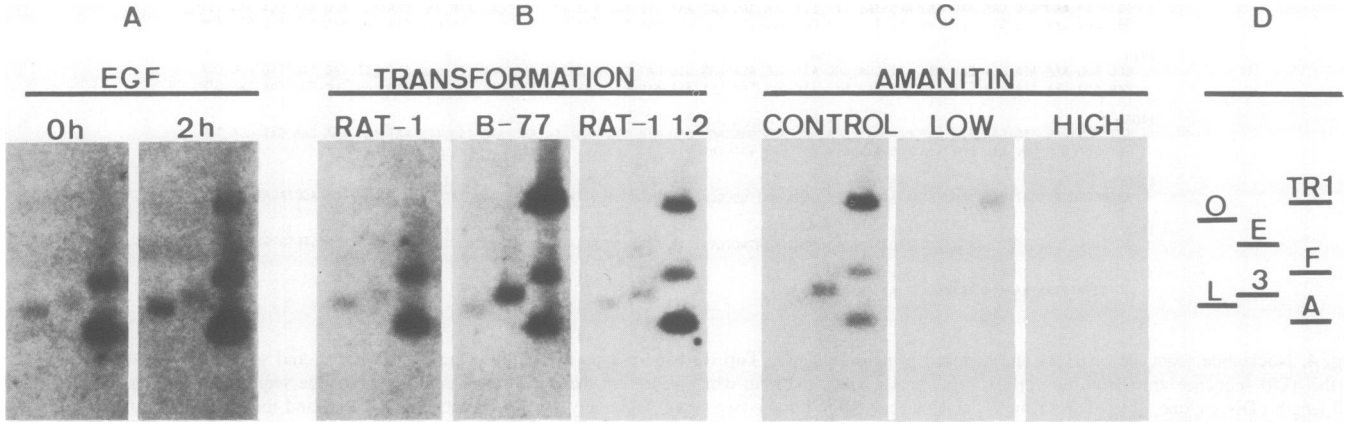

Fig. 6. Nuclear run-on transcription assays. (**A**) Nuclei were isolated from Rat-1 cells with or without a 2 h EGF treatment as described in the legend to Figure 2. 1 x 10^7 c.p.m. were hybridized to each filter. (**B**) Nuclei were isolated from Rat-1, B-77, or Rat-1 1.2 cells that were exposed to serum-free medium for 24 h. 2.7 x 10^6 c.p.m. were hybridized to each filter. (**C**) Nuclear run-on transcriptions were carried out using nuclei from B-77 cells (in serum free medium) in the presence of 0 (control), 1 (low), and 200 (high) μg/ml of α-amanitin. 1.3 x 10^6 c.p.m. were added to each filter. (**D**) Schematic representation of DNA fragments bound to each filter. Each filter contains the following restriction enzyme fragments from the indicated plasmids. pTR1: 1.6-kb *Eco*RI fragment from pTRl; F: 0.9-kb *Eco*RI fragment from pF (see legend to Figure 1). **A**: 0.5-kb *Kpn*I-*Bgl*II fragment from pACT containing rat actin sequence (Matrisian *et al.*, 1985); **E**: 1.0-kb *Eco*RI fragment from pUNENK containing rat preproenkephalin sequences (L.Matrisian, G.Rautmann, R.Breathnach, unpublished); **3**: 0.7-kb *Eco*RI fragment from pUNre3.1, a random clone selected from the cDNA library described in this work; **0**: 1.8-kb *Bam*HI fragment from pFLAG containing chicken ovalbumin cDNA sequences (R.Breathnach, unpublished); **L**: 0.6-kb *Pvu*II fragment from pLDH containing rat lactate dehydrogenase sequences (Matrisian *et al.*, 1985). pBR322 DNA was also included on gel filters and no signal was observed (data not shown).

control DNA fragments either give no signal (pBR322, chicken ovalbumin cDNA, or rat preproenkephalin cDNA) or a roughly constant signal (rat lactate dehydrogenase cDNA, rat actin cDNA and two random cDNAs). In addition, the pTR1 RNA transcription observed in nuclei from B-77 cells can be almost completely blocked by 1 μg/ml actinomycin D (Figure 6), as is also the case for the lactate dehydrogenase or actin gene transcription, showing that the pTR1 gene is transcribed by RNA polymerase B (or II). We conclude from the above results that control of pTR1 RNA levels by both oncogenes and EGF occurs at the transcriptional level, and that EGF induces transcription of the pTR1 gene within 2 h (shortest time of treatment tested).

Discussion

We have described a novel cellular mRNA whose transcription is induced by transformation with polyoma middle T, *src*, and H-*ras* oncogenes and also by the polypeptide growth factor EGF. This material should prove particularly useful for investigations of the modes of action of oncogenes and growth factors, and points already to the existence of an at least partially overlapping mechanism of action of these agents in rat fibroblasts.

What features do the proteins capable of inducing transcription of the pTR1 gene have in common? Polyoma middle T protein, and the protein products of the *src* and H-*ras* oncogenes, as well as a domain of the EGF receptor are positioned on the cytoplasmic side of the plasma membrane (Hunter, 1984) and might therefore interact with one another. Polyoma middle T protein has indeed been shown to interact with the protein encoded by the cellular *src* gene (Bolen *et al.*, 1984; Courtneidge and Smith, 1984) and there is some evidence for an interaction between the EGF receptor and the H-*ras* gene product (Kamata and Feramisco, 1984). The *ras* gene product itself has been linked to the adenylate cyclase signalling system in yeast (Toda *et al.*, 1985). Activation of the pTR1 gene transcription by EGF or the H-*ras* oncogene could perhaps pass *via* the adenylate cyclase system in rat cells.

It is also interesting to note that, of the agents that have been shown to induce the pTR1 RNA, the *src* (Bishop, 1983) and polyoma middle T (Eckhart *et al.*, 1979; Courtneidge and Smith, 1984) oncogenes and the EGF receptor (Ushiro and Cohen, 1980) are associated with tyrosine kinase activity, although there is no evidence that the *ras* oncogene possesses this activity (Bishop, 1983). Another feature of cells transformed by RSV, *ras* and polyoma virus is their ability to secrete TGF-like factors (Roberts *et al.*, 1983; Kaplan and Ozanne, 1982; Anzano *et al.*, 1985). EGF itself is considered to be a TFG-α and potentiates TGF-β activity (Roberts *et al.*, 1983). Further studies will elucidate whether tyrosine kinase activity or TGFs play a role in the control of pTR1 RNA production. (Our initial experiments with PyT21-conditioned medium, however, showed no increase in pTR1 RNA levels.)

Recently, a model has been proposed that suggests that the proliferative effects of oncogenes (including the *src* and *ras* oncogenes) and growth factors (including EGF) are mediated in part *via* membrane phosphatidylinositol lipids with production of the second messengers diacylglycerol and inositol triphosphate ($InsP_3$) (Berridge and Irvine, 1984). Treatment of Rat-1 cells for 24 h with the phorbol ester TPA, which mimics the activating effects of diacylglycerol on protein kinase C, did not stimulate the production of pTR1 mRNA. This observation suggests that the stimulation of pTR1 mRNA by EGF is not mediated by protein kinase C, although further studies are necessary to determine what role, if any, these intracellular pathways play in the induction of pTR1 RNA.

We have previously shown that EGF treatment of quiescent Rat-1 cells results in an increase in mRNA levels for four glycolytic enzymes and an actin (Matrisian *et al.*, 1985). These RNAs, as well as others that are induced by the growth factors EGF (Muller *et al.*, 1984) or PDGF (Muller *et al.*, 1984; Linzer and Nathans, 1984; Cochran *et al.*, 1983), are also induced by serum. pTR1 RNA, in contrast, is not present in serum-stimulated cells and its expression can in fact be inhibited by serum. Thus, the pTR1 system may provide a unique opportunity to examine events that are activated in response to stimulation by certain oncogenes and growth factors but are not necessarily part of a 'normal' growth response. The pTR1 system is rendered even more attractive since its regulation is at the transcriptional level. Identification of the pTR1 protein and further studies on events that modulate its level should provide exciting insights into the control mechanisms that function in transformed cells in culture and therefore possibly in carcinogenic cells *in vivo*. In this respect it is interesting that sequences homologous to rat pTR1 exist in human genomic DNA (our unpublished observations).

Materials and methods

Differential screening

A cDNA library was made from poly(A)$^+$ RNA obtained from the PyT21 cell line using the λgt10 cloning system as described elsewhere (Huynh *et al.*, 1985). For preparations of enriched probes (Hedrick *et al.*, 1984), ^{32}P-labelled cDNA reverse transcribed from 2 μg of PyT21 cell poly(A)$^+$ RNA was annealed overnight at 68°C to 16 μg of FR3T3 cell poly(A)$^+$ RNA in a volume of 8 μl containing 0.5 M sodium phosphate ($NaPO_4$) buffer pH 7.0, 1 mM EDTA and 0.25% SDS in a sealed siliconised glass capillary. This mix was diluted into 1 ml 0.12 M $NaPO_4$ buffer containing 0.1% SDS and passed over a 500 μl hydroxylapatite (Biorad) column equilibrated and run at 60°C. The column was washed with the same buffer, and eluate fractions containing radioactivity were pooled, ethanol precipitated and desalted on a 1 ml G50 column. Radioactive material in the eluate was precipitated with ethanol in the presence of 16 μg of FR3T3 poly(A)$^+$ RNA, and the hybridization-subtraction procedure repeated. The eluate from the second hydroxylapatite column was used for screening 20 000 recombinant phage bound to eight nitrocellulose filters. Hybridization was carried out at 42°C for 72 h in 5 x SSC, 1 x Denhardts solution, 200 μg/ml denatured salmon sperm DNA containing 50% formamide. Filters were washed with 2 x SSC at 68°C. Positively responding phages were isolated and their *Eco*RI inserts transferred to pUN121 (Nilsson *et al.*, 1983) using standard techniques (Maniatis *et al.*, 1982).

DNA sequencing

Sequences were determined using either the Maxam and Gilbert (1980) technique, or the M13-dideoxy technique (Sanger *et al.*, 1977; Messing, 1983) with [^{35}S]dATP and buffer gradient gels (Biggin *et al.*, 1983). A 'full-length' pTR1 cDNA was isolated from a cDNA library prepared from PyT21 cell RNA using the Okayama and Berg (1982) technique as described previously (Breathnach and Harris, 1983). A genomic DNA sequence complementary to the pTRl cDNA insert was obtained by screening 300 000 recombinant phage carrying 15–20 kb fragments of Rat-1 cell DNA in the vector EMBL3 (Frischauf *et al.*, 1983). Sequences complementary to the 5′ end of the pTR1 cDNA insert were localised to a 1.1-kb *Bam*HI-*Pst*I fragment which was sequenced from its *Pst*I terminus by the Maxam and Gilbert technique.

Cell culture

Tissue culture cells were maintained in 10% fetal calf serum at 37°C in a 95% air/5% CO_2 atmosphere. The derivation of the various cell lines used in these experiments is described in the following references: normal rat fibroblast line FR3T3 (Seif and Cuzin, 1977), polyoma virus-transformed FR3T3 line PyT21 (Rassoulzadegan *et al.*, 1982), polyoma middle T-transformed FR3T3 line MTT4 (Rassoulzadegan *et al.*, 1982), RSV-transformed FR3T3 line FR-RSV (a kind gift of F.Cuzin), normal rat fibroblast line Rat-1 (Prasad *et al.*, 1976), RSV-transformed-Rat-1 line B-77 (Magun *et al.*, 1979) and polyoma middle T-transformed-Rat-1 line Rat-1 1.2 (Treisman *et al.*, 1981). LA-24 cells are Rat-1 fibroblasts transformed by a temperature-sensitive mutant of RSV (Magun *et al.*, 1979). The FRras1 and RTras1 and RTras2 lines were derived from FR3T3 or Rat-1 cells, respectively, by transformation with the cellular oncogene H-*ras* from EJ bladder cancer cells (N.Glaichenhaus, unpublished).

Northern blots

Cytoplasmic RNA was isolated from cultured cells as previously described (Masiakowski *et al.*, 1982; Matrisian *et al.*, 1985). The RNA (10 μg) was electrophoresed on a 1.5% agarose gel containing 2.05 M formaldehyde in a buffer containing 20 mM Mops, 3 mM sodium acetate and 1 mM EDTA, pH 7.6. The RNA was transferred to nitrocellulose paper and hybridized to nick-translated probes (Breathnach *et al.*, 1977) in the presence of 50% formamide, 5 x SSC, 1 x Denhardts solution, 20 mM sodium phosphate, 0.1% SDS, 50 μg/ml salmon sperm DNA and 4% dextran sulfate at 42°C. The filters were washed in 0.1 x SSC and 0.1% SDS at 50°C and exposed to X-ray film using intensifying screens.

Nuclear run-on transcription

Nuclei were pelleted from tissue culture cells after lysis in a hypotonic solution containing 0.5% NP40 (Masiakowski *et al.*, 1982) and frozen at −80°C in an equal volume of 40% glycerol, 50 mM Tris, 5 mM $MgCl_2$ and 0.1 mM EDTA. The run-on transcription was carried out in the presence of 120 mM Tris-HCl pH 7.8, 50 mM NaCl, 350 mM ammonium sulfate, 4 mM manganese chloride, 0.24 mM EDTA, 1 mg/ml heparin and 1 mM each of ATP, GTP and UTP. 10−25 μCi of [α-^{32}P]CTP (Amersham, 400 Ci/mmol) was added to a reaction mixture containing 40 μl of nuclei in 100 μl final volume and the mixture was incubated at 32°C for 45 min (Brown *et al.*, 1984). DNA and protein was then removed from the sample by digestion with DNase I and proteinase K, by taking advantage of the observation that proteinase K digests RNase but not DNase in the presence of $CaCl_2$ (Tullis and Rubin, 1980; P.Masiakowski, in preparation). DNase I and proteinase K (25 μl of a 1 mg/ml solution of each in 20 mM Tris-HCl, 10 mM $CaCl_2$) were pre-incubated at 37°C and added to the run-on transcription sample that was diluted by the addition of 350 μl of 20 mM Tris-HCl, 10 mM $CaCl_2$ and 10 μg of tRNA carrier. The solution was incubated at 37°C for 30 min, followed by the addition of 50 μl of 0.2 mM EDTA and 50 μl of 10% SDS and further incubation at 37°C for 15 min. The sample was extracted with phenol-chloroform and precipitated by the addition of an equal volume (600 μl) of cold 20% TCA. After precipitation on ice for 15 min, the sample was centrifuged in a microfuge for 15 min and the pellet was washed four times in ice-cold 5% TCA. The pellet was dissolved in 25 mM Tris-HCl, 1 mM EDTA, adjusted to 0.3 M sodium acetate, and ethanol precipitated. The RNA was then reconstituted in H_2O and an aliquot was counted. Within an experiment, an identical amount of radioactivity was added for each filter and hybridized for 4 days at 42°C in the presence of 50% formamide (Brown *et al.*, 1984). The filters were prepared by electrophoresis of purified restriction enzyme fragments from the indicated plasmids on a 1.2% agarose gel and blotting onto DBM paper (Gariglio *et al.*, 1981). Filters were washed in 0.1 x SSC and 0.1% SDS at 42°C and exposed to Kodak XAR-5 film using intensifying screens.

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