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Isolation of the Oncogene and Epidermal Growth Factor-Induced Transin Gene: Complex Control in Rat Fibroblasts

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Various oncogenes or epidermal growth factor (EGF) induce transcription of a 1.9-kilobase RNA (transin RNA) in rat fibroblasts. The induction by EGF can be blocked by cycloheximide. Thus the response of the transin gene to EGF appears to require de novo protein synthesis. Transin RNA induction is specific to EGF, as neither insulin, platelet-derived growth factor, fibroblast growth factor, nor transforming growth factor β could elicit the same response. However, transforming growth factor β could block the EGF induction of transin RNA. Whereas the calcium ionophore A23187 and the tumor promoter TPA, either alone or administered together, did not increase transin RNA levels, TPA could synergise with a serum factor to effect such an increase. Dibutyryl cyclic AMP also induced transin RNA. Treatment of cells with the microfilament-disrupting agent cytochalasin B, but not the microtubule-disrupting agent colcemid, resulted in an increase in transin RNA levels, suggesting a role for the cytoskeleton in control of transin gene expression. The transin RNA does not contain repeated sequences and appears to be encoded by a single-copy gene. The protein sequence encoded by the last four exons of the transin gene shows some homology to two regions of the heme-binding protein hemopexin.

Detailed knowledge of the mechanisms of action of oncogenes, growth factors, and growth factor receptors is important as a means of furthering our understanding of the loss of growth control characteristic of the neoplastic state. The recent discoveries of structural and functional similarities between certain oncogene products and growth factors or growth factor receptors has opened avenues of research that continually reveal new links between the action of oncogenes and growth factors (9, 21). Thus the recent finding that the c-*fms* proto-oncogene product is related to the receptor for the mononuclear phagocyte growth factor colony stimulating factor (CSF-1) (49) joins the previous observations that the cellular homolog of the oncogene v-*sis* is a gene encoding platelet-derived growth factor (PDGF; 12, 59) and that the product of the retroviral oncogene v-*erbB* appears to be a truncated version of the cellular epidermal growth factor (EGF) receptor, lacking the EGF binding domain (13). While these findings show that some oncogenes may function by imitating growth factors or occupied growth factor receptors, little is known about how the growth factor/receptor interaction itself leads to accomplishment of the complex events involved in cell division.

One of the most studied growth factor/receptor systems is the interaction between EGF and its receptor at the cell surface. This interaction activates the receptor's tyrosine kinase activity (58) and leads to a rapid increase in intracellular calcium levels and pH (22, 36). Another consequence of EGF treatment of a variety of cell types is a rapid increase in levels of c-*myc*, c-*fos*, and actin mRNAs (8, 15, 33, 37). This increase, a result either of a decreased rate of mRNA degradation (c-*myc*; 7) or an increased rate of mRNA production (c-*fos* and actin), is a primary response to EGF treatment insofar as it is not blocked by protein synthesis inhibitors (15, 37). It seems likely that the transcription of other genes would be necessary for the expression of the full response to the growth factor. These genes might be expected to be expressed constitutively in transformed cells. The study of such genes should provide new insights into the mechanisms used by growth factors and oncogenes to effect or override the control of cell division.

We have previously described the isolation and characterization of an mRNA that is present in significantly higher levels in rat fibroblasts transformed by polyomavirus, Rous sarcoma virus, or the cellular oncogene h-*ras* than in the normal parental cell line (32). In addition, this mRNA, referred to previously as pTR1 RNA and which we now term transin RNA, is elevated in Rat-1 cells upon stimulation with EGF. We show here that de novo protein synthesis is required for EGF induction of transin RNA, establishing the transin gene as a "secondary response" gene. Expression of transin RNA is under complex control in rat fibroblasts, and we discuss a possible role for the cytoskeleton in this control. We also describe the structure of the transin gene and show that the gene is distantly related to that of a heme-binding protein.

MATERIALS AND METHODS

Materials. Cytochalasin B, colcemid (demecolcine), dibutyryl cyclic AMP (dibutyryl cAMP), dibutyryl cyclic GMP (dibutyryl cGMP), 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), insulin, and cycloheximide were obtained from Sigma Chemical Co., St. Louis, Mo. The ionophore A23187 was from Calbiochem, La Jolla, Calif.; PDGF and platelet-poor plasma were obtained from Biomedical Technologies Inc., Cambridge, Mass. EGF and transforming growth factor β (TGF-β) were kind gifts from Bruce Magun. The EGF used was isolated from mouse submaxillary glands and purified to the BioGel P-10 step as described by Savage

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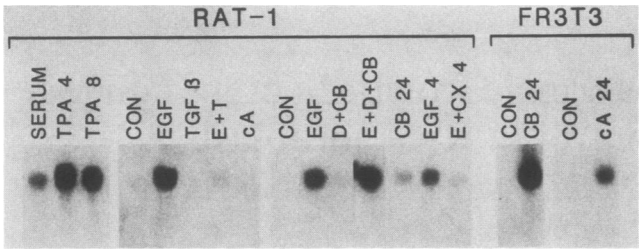

FIG. 1. Northern analysis of transin RNA levels. Serum-starved Rat-1 cells were exposed in medium for 8 h to 10% fetal calf serum (serum), 10% fetal calf serum containing TPA (TPA 8), EGF (EGF), TGF-β (TGF β), EGF plus TGF-β (E+T), colcemid plus cytochalasin B (D+CB), EGF plus colcemid plus cytochalasin B (E+D+CB), or medium alone (CON). TPA 4: Rat-1 cells were exposed to 10% fetal calf serum for 8 h with TPA present for the last 4 h. cA, cA 24: Cells were exposed to dibutyryl cAMP for 24 h in serum-free medium. CB 24: Cells were exposed to cytochalasin B for 24 h. EGF 4, E+CX 4: Cells were exposed to EGF for 4 h in the presence (E+CX 4) or absence (EGF 4) of cycloheximide.

and Cohen (45). The TGF-β was isolated from outdated human platelets and purified to the urea-containing BioGel P60 step as described by Assoian et al. (1). Fibroblast growth factor (FGF) was kindly provided by G. Labourdette.

Cell culture. Cultured cells were maintained in Dulbecco modified Eagle medium containing 10% fetal calf serum and appropriate antibiotics at 37°C in a 95% air–5% CO_2 atmosphere. Normal Rat-1 and FR3T3 fibroblasts (40, 48) were obtained from B. Magun and F. Cuzin, respectively. For analysis of transin RNA levels, cells were washed once in phosphate-buffered saline shortly after reaching confluence (≤12 h), and the medium was replaced with serum-free medium overnight. The various biological modifiers were added directly to the serum-free medium unless otherwise stated. Concentrations used were: TPA, 100 ng/ml; EGF, 2 ng/ml; TGF-β, 5 ng/ml; dibutyryl cAMP, 0.5 mM; colcemid, 2 μM; cytochalasin B, 5 μg/ml; cycloheximide, 10 μg/ml; FGF, 20 ng/ml; insulin, 1 μg/ml; PDGF, 20 ng/ml; platelet-poor plasma, 5%.

RNA and DNA analyses. Total cytoplasmic RNA was isolated from cultured cells by Nonidet P-40 lysis and electrophoresed on a 1.5% agarose gel containing 2.05 M formaldehyde as previously described (32). The RNA was transferred to nitrocellulose and hybridized at 42°C in the presence of 50% formamide to nick-translated probes as previously described (32). Rat-1 cellular DNA was isolated as described by Maniatis et al. (29) and completely digested with restriction enzymes. The DNA fragments were separated by electrophoresis on a 1.2% agarose gel and blotted onto nitrocellulose filters as described (29). The filters were hybridized with the nick-translated transin probe pTR1 (32) in the presence of 50% formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1× Denhardt solution–20 mM sodium phosphate–0.1% sodium dodecyl sulfate–50 μg of salmon sperm DNA per ml–4% dextran sulfate at 42°C. The filters were washed in 0.1× SSC–0.1% sodium dodecyl sulfate at 50°C and exposed to X-ray film using intensifying screens. The human DNA blot was a gift of J. P. Moisan. Hybridization conditions were as described above for the rat DNA filters except that the formamide was at 40% and the washing conditions were 2× SSC–0.1% sodium dodecyl sulfate at 50°C.

Analysis of the genomic transin gene. Recombinant EMBL 3 phage carrying fragments of rat genomic DNA complementary to the transin cDNA in pTR1 were isolated as described previously (18, 32). For studies of the gene by sequencing or electron microscopy, fragments of two of the four phages obtained were subcloned into pBR322 as described in the text and the legend of Fig. 3, using standard techniques. Thus a fragment of phage 11 extending from *Bam*HI site B1 to the vector *Sal*I site marking the end of the rat DNA insert in phage 11 was introduced between the *Bam*HI and *Sal*I sites of pBR322, and a fragment of phage 21 extending from the *Eco*RI site E6 to the vector *Sal*I site marking the end of the rat DNA insert in phage 21 was introduced between the *Eco*RI and *Sal*I sites of pBR322. The resulting plasmids were linearized with *Sal*I and hybridized to a linearized plasmid containing a full-length transin cDNA insert obtained previously (32). Hybrids were prepared for electron microscopy as described previously (19). The electron microscopy results led to a preliminary map of the gene structure that was confirmed by restriction enzyme mapping and limited nucleotide sequencing. Thus the positions of exons 1, 3, 4, 6, 7, and 8 were verified by sequencing from sites corresponding to sites present in the cDNA (see Fig. 3), namely, a *Pst*I site for exon 1, a *Hin*dIII site for exons 3 and 4, a *Bgl*II site for exon 6, a *Bam*HI site for exon 7, and *Pvu*II and *Hin*cII sites for exon 8. The data obtained showed that exons 3 and 4 contain sequences contiguous in the cDNA, as do exons 6, 7, and 8. Furthermore, exon 8 contains the sequences encoding the very 3′ end of transin RNA, and exon 1 contains those encoding its very 5′ end.

Nucleotide sequencing. Sequencing was carried out using either the Maxam and Gilbert (34, 35) or dideoxy (44) techniques with buffer gradient gels (5). The sequence of the *Bam*HI-*Pst*I fragment shown by arrow 3 in Fig. 3 was obtained by the dideoxy technique on fragments generated by random sonication and introduced into M13tg 130 (24). Further sequence was obtained using the same vector and *Sau*3AI or *Hae*III fragments of the *Bam*HI-*Pst*I fragment.

RESULTS

We have shown previously that transin RNA is induced in rat fibroblasts by the action of various oncogenes or EGF (32). We wished to investigate this induction further and in particular to address the following questions: (i) is transin RNA induction a primary response to EGF? (ii) can other growth factors play a role, positive or negative, on the control of transin RNA expression? (iii) can the induction of transin RNA be effected by any of the common second messengers? For these experiments, Rat-1 fibroblasts were serum starved overnight and exposed to various agents in serum-free medium (unless otherwise stated). Cytoplasmic RNA was extracted and analyzed by Northern blotting and hybridization to the nick-translated transin probe (Fig. 1).

Protein synthesis requirement for EGF induction. In confirmation of our previous work, exposure of Rat-1 cells to EGF for 4 h resulted in a clear induction of transin RNA. However, the addition of the protein synthesis inhibitor cycloheximide to Rat-1 cells at the same time as EGF abolished this induction (Fig. 1; compare lanes EGF 4 and E+CX 4). To determine whether this effect was due to a general decrease in transcription caused by the presence of the protein synthesis inhibitor, we performed nuclear run-on transcription assays on nuclei isolated from cells exposed to EGF in the presence or absence of cycloheximide for 4 h. Cycloheximide blocked the transcription of transin RNA, but did not alter transcription of actin RNA, of lactate dehydrogenase RNA, or of a control RNA referred to as F (32) (data not shown). Cycloheximide therefore specifically blocked EGF induction of transin RNA without lowering

general transcriptional activity under these conditions. Thus, in contrast to some EGF-inducible genes already described in the literature (15, 37), the induction of the transin gene by EGF requires the de novo synthesis of a protein or proteins.

Specificity for EGF. To determine whether the induction of transin RNA in Rat-1 cells is limited to EGF or can be reproduced by other growth factors, we exposed Rat-1 cells to PDGF in the presence or absence of platelet-poor plasma, FGF, insulin, or TGF-β. None of these factors stimulated transin RNA in Rat-1 cells after 8 or 24 h of exposure (data not shown). However, whereas TGF-β alone did not alter transin RNA levels (Fig. 1, lane TGF-β, and data not shown), when EGF and TGF-β were added together TGF-β blocked the EGF induction of transin RNA (Fig. 1; compare lanes EGF and E+T). We have observed this inhibitory effect of TGF-β with two different preparations of TGF-β from human platelets, and while the concentration of TGF-β necessary for this effect varied with the two different preparations, it did reflect the purity of the TGF-β preparation. At this time, however, we cannot entirely rule out the possibility that a minor contaminant of the TGF-β preparation is responsible for blocking EGF induction of transin RNA.

The induction of transin RNA in Rat-1 cells thus appears specific to EGF. We had shown previously that a factor or factors present in some batches of serum could block this induction (32). Our present results show that a purified growth factor, TGF-β, may be responsible for negative control of transin RNA levels.

Effects of second messengers. Recently the role of diacylglycerol and inositol triphosphate as second messengers which effect, respectively, activation of protein kinase C and mobilization of intracellular calcium stores has come under intensive study in relation to the mechanism of action of growth factors and some oncogenes (4, 39). The phorbol ester TPA mimics the effects of diacylglycerol on protein kinase C (4, 39). However, TPA is not effective in inducing transin RNA in Rat-1 or FR3T3 cells in serum-free medium (32) or in the presence of 0.1% bovine serum albumin (data not shown); the latter experiment rules out a simple need for a carrier protein to prevent nonspecific adsorption of the phorbol ester. Nevertheless, protein kinase C may play a role in controlling transin RNA levels, as TPA, in the presence of fetal calf serum, will significantly increase the levels of transin RNA seen in the presence of serum alone. (Compare lanes labeled serum, TPA 4, and TPA 8 in Fig. 1. In these experiments, the fetal calf serum weakly induces transin RNA, an observation that varies depending on the batch of fetal calf serum used, as we have previously reported an inhibition of transin RNA levels by other batches of serum [32]. Doubtless, the growth factor content of different batches of serum will differ, and a given batch may be more or less rich in factors capable of inducing or repressing transin RNA levels.) This conclusion is strengthened by the fact that TPA in serum-free medium will induce transin RNA (data not shown) in the mouse epidermal cell line MCA3d (26).

The calcium inophore A23187 allows an influx of extracellular calcium and therefore mimics one effect of EGF (22) and at least some of the effects of the second messenger inositol triphosphate, which induces a release of calcium from intracellular stores (4). Addition of the ionophore alone (100 nM) had no effect on transin RNA levels after 8 or 24 h of exposure (data not shown). The combination of ionophore (100 nM) and TPA (100 ng/ml) also had no effect on transin RNA levels (data not shown). Addition of the ionophore (10 to 100 nM) concomitantly with EGF had no effect on the EGF induction of transin RNA, although at relatively high concentrations of ionophore (1 to 10 μM) the transin RNA signal was eliminated, presumably due to a toxic effect of the ionophore after 8 h of exposure (data not shown). These data indicate that an elevation in intracellular Ca^{2+}, either alone or in conjunction with activation of protein kinase C by TPA, is not sufficient to induce transin RNA in Rat-1 cells. We have also tested the effect of the classic second messengers cAMP and cGMP on transin RNA levels in rat fibroblasts. The cAMP analog dibutyryl cAMP had no effect on transin RNA levels in Rat-1 fibroblasts upon 8 or 24 h of exposure (Fig. 1, lane cA). In contrast, transin RNA was just apparent in FR3T3 fibroblasts after 4 h of dibutyryl cAMP exposure and was markedly elevated at 24 h (Fig. 1, lane cA24). We have observed a number of other differences between these two fibroblastlike cell lines, including different responses to EGF (33) and a tendency of the FR3T3 cells, but not the Rat-1 cells, to express preproenkephalin mRNA (unpublished data). Their different responses to cAMP are therefore not surprising. The addition of dibutyryl cGMP to Rat-1 or FR3T3 cells for either 8 or 24 h resulted in no elevation in transin RNA levels (data not shown).

Induction after microfilament disruption. A disruption of the cytoskeleton is a characteristic shared by many transformed cell lines (3), and EGF has been reported to lead to modifications in the cytoskeletal architecture in some cell lines (46). It was thus of interest to determine whether disruption of the cytoskeleton would have any effect on transin RNA levels in rat fibroblasts. We tested the effects of both cytochalasin B, a microfilament-disrupting agent, and colcemid, a microtubule-disrupting agent.

Cytochalasin B induced transin RNA in both Rat-1 and FR3T3 fibroblasts after 24 h of exposure (Fig. 1, CB 24). The effect of cytochalasin B is relatively slow, however, as transin RNA was not evident in Rat-1 cells after cytochalasin B treatment for 8 h (data not shown, but see lane D+CB, i.e., colcemid plus cytochalasin B). Colcemid had no effect on transin RNA levels in Rat-1 cells after either 8 or 24 h of exposure (data not shown). Interestingly, we have observed that the induction of transin RNA by EGF is not altered by the presence of both colcemid and cytochalasin B in an 8-h incubation, suggesting that an intact cytoskeleton is not necessary for EGF induction of transin RNA (compare lanes EGF and E+D+CB in Fig. 1).

The time courses of induction of transin RNA by the various stimulatory agents were markedly different: EGF (32) and the combination of TPA and serum increased transin RNA levels by 4 h, cAMP-induced transin RNA was barely detectable at 4 h, and cytochalasin B required ≥8 h of exposure before an elevation in transin RNA levels was observed (see Fig. 1). The effects of EGF and TPA-serum on transin levels precede their effect on DNA synthesis, as neither of these agents causes significant increases in the percent of DNA replicated in these cells until after 10 h of exposure (28, 31). Transin induction is not, however, obligatory for the proliferative response, as serum stimulation of quiescent Rat-1 cells does not induce transin RNA, and serum in fact has an inhibitory effect on transin RNA levels (32).

Isolation of a gene encoding transin RNA. Many of the oncogene- or growth factor-induced RNAs identified by differential screening of cDNA libraries either contain repeated sequences or are themselves repeated sequences (16, 38, 52, 62). It was thus important to determine whether

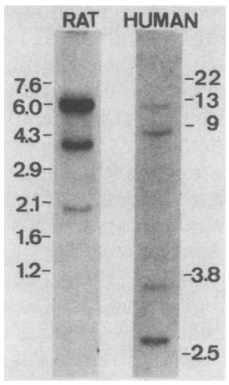

FIG. 2. Southern analysis of *Eco*RI-digested rat and human DNA. Size markers were pBR322 and λ DNA digests. Rat bands correspond in order of decreasing size to fragment E1-E2, a comigrating fragment from E6 to a downstream *Eco*RI site not shown, fragment E5-E6, and fragment E2-E3 (see Fig. 3).

full-length transin cDNA contained repeated sequences. Furthermore, as the transin gene is regulated at the transcriptional level by EGF and oncogenes, it was of interest to investigate the 5′ end of the gene in the putative promoter region for comparison with promoter regions of other growth factor-induced genes.

We have previously described the isolation of a full-length transin cDNA by the Okayama and Berg technique (32). Primer extension studies (not shown) have confirmed that this cDNA is indeed full length. Rat-1 cellular DNA was examined for transin sequences by Southern analysis. Hybridization of the nick-translated full-length transin cDNA probe to *Eco*RI fragments of rat or human DNA revealed a small number of bands (Fig. 2). This suggests that there is at most a limited number of transin genes in rat or human DNA and that the transin mRNA may be the product of a single-copy gene. Further evidence that the transin gene is a single gene came from the isolation and characterization of this gene. Rat genomic DNA complementary to the transin cDNA was obtained by screening 5×10^5 recombinant phage carrying 15- to 20-kilobase fragments of Rat-1 cellular DNA in the lambda phage vector EMBL3 (18). Four phage gave a positive signal with the transin probe. Restriction enzyme mapping of their rat DNA inserts showed that they contained overlapping regions of the same gene (see arrows 11 and 21 in Fig. 3 for the extent of two of these phage inserts). To facilitate characterization of the gene, an 11-kilobase fragment (arrow 2, Fig. 3) spanning the *Bam*HI site B1 to the end of phage 11 (a *Sal*I site in the vector) and a 6.0-kilobase fragment (arrow 1) spanning the *Eco*RI site E6 to the vector *Sal*I site of phage 21 were subcloned into pBR322. These pBR322 recombinants were linearized at their *Sal*I sites and hybridized to a linearized plasmid carrying a full-length transin cDNA, and samples were prepared for electron microscopy (Fig. 4). On the basis of this information, together with restriction enzyme mapping and sequencing data, a map of the rat transin gene was constructed, revealing eight exons spanning a total of ~14 kilobases (Fig. 3). As described in the legend to Fig. 2, all the bands seen by Southern blotting in an *Eco*RI digest of Rat-1 DNA can be accounted for on the basis of one transin gene with the structure shown in Fig. 3, although we cannot exclude rigorously the existence of distantly related genes.

LINE element close to the cap site. An extensive area upstream of exon 1 of the rat transin gene was sequenced (Fig. 5; *Bam*HI-*Pst*I fragment; see arrow 3 in Fig. 3). As reported previously (32), the region directly 5′ to the cap site is characterized by the presence of a classic TATA box at position −30 and a CAAT box at position −77 relative to the mRNA start site as determined by primer extention studies. However, no other clear homologies with the promoter elements of other EGF-induced genes, i.e., c-*fos*, prolactin, and VL30 (sequences taken from the European Molecular Biology Laboratory, Heidelberg, Federal Republic of Germany [EMBL], data bank), could be detected.

A comparison of the sequence shown in Fig. 5 with those contained in the EMBL data bank revealed the presence of a repetitive element of the R family (part of the LINE family; 51) running from the *Bam*HI site at position 1 to the run of A residues at position 516, and thus ending 591 nucleotides upstream of the cap site (see Fig. 5).

Partial transin-hemopexin homology. We have published previously (32) the sequence of transin RNA and of the corresponding protein transin (as deduced from the nucleotide sequence). At the time, screening of data banks showed no homologous sequence. We have recently rescreened the National Biomedical Research Foundation, Bethesda, Md. [NBRF], data bank with the transin sequence and discovered a homology with the protein hemopexin (Fig. 6). Hemopexin is a heme-binding plasma protein consisting of two similar halves (55). In the alignment shown in Fig. 6A there are 58 identities out of 174 possible matches, with 17 breaks, often single residue deletions. For comparison, the internal homology of hemopexin gives 56 identities out of 172 possible matches with 13 breaks, generally extensive deletions. Residues 263 through 463 of transin are 28% homologous to residues 4 through 193 of hemopexin (Fig. 6A). Residues 281 through 412 of transin are 25% homologous to residues 228 through 363 of hemopexin (Fig. 6B). The internal homology of hemopexin covers residues 1 through 216 and 217 through 439. We have determined the nucleotide sequence surrounding the intron-exon junction marking the start of exon 4 as 5′-CCCTTTAG↓GA/CCT/CCC/-3′, where the arrow marks the splice point and the dashes indicate the reading frame. The two proline residues 263 and 264 of transin, which mark the beginning of the transin-hemopexin homology shown in Fig. 6A, are thus the very first amino acids encoded by exon 4 of the transin

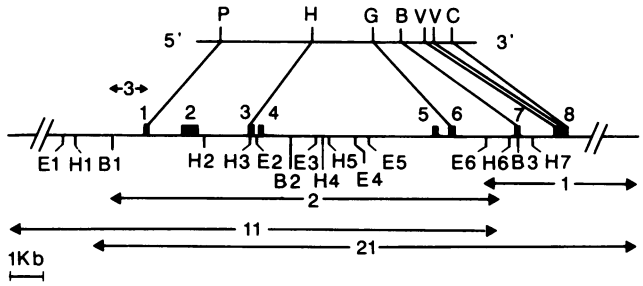

FIG. 3. Map of the rat transin gene. The rat transin cDNA is shown with sites for the enzymes *Pst*I (P), *Hin*dIII (H), *Bgl*II (G), *Bam*HI (B), *Pvu*II (V), and *Hin*cII (C). Lines link these sites to exons (solid boxes) on the transin gene map. Shown beneath the gene map are sites for enzymes *Eco*RI (E), *Bam*HI (B), and *Hin*dIII (H). Arrow 1, *Eco*RI-*Sal*I fragment extending from site E6 to a vector *Sal*I site marking the end of rat DNA in phage 21. Arrow 2, *Bam*HI-*Sal*I fragment extending from site B1 to a vector *Sal*I site marking the end of rat DNA in phage 11. Arrow 3, *Bam*HI-*Pst*I fragment whose sequence appears in Fig. 5. Arrows 11 and 21, Extent of phages 11 and 21. The scale (Kb, kilobase) refers to the transin gene map.

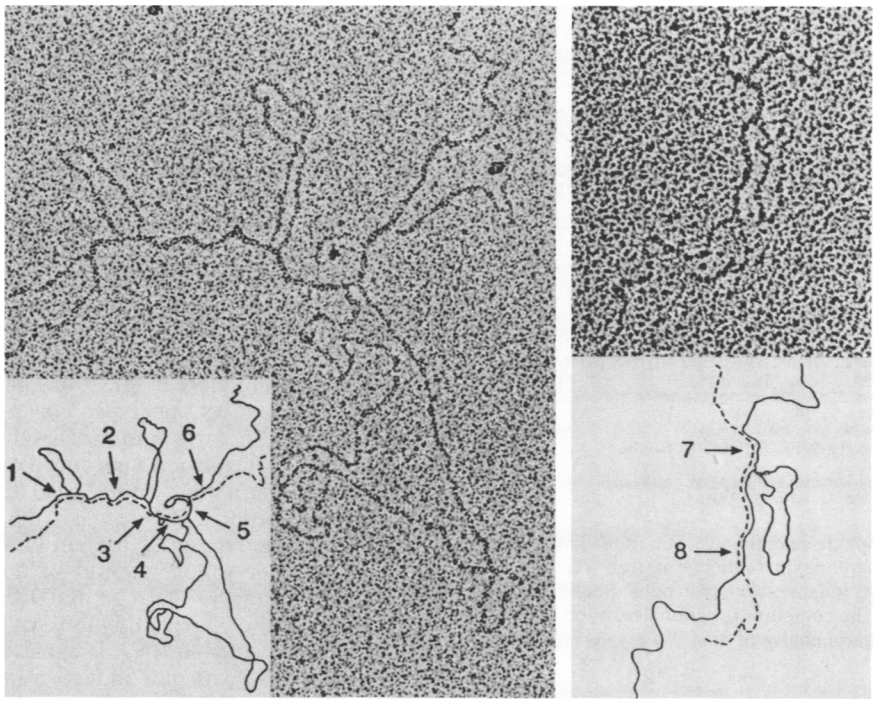

FIG. 4. Electron microscopy of transin gene-cDNA hybrids. A plasmid containing a full-length transin cDNA was hybridized with plasmids containing the fragments of the gene defined by arrows 1 (right panel) or 2 (left panel) of Fig. 3. In the schematic representations, the plasmid containing the cDNA is shown as a dotted line, and the plasmid containing the gene fragments is indicated as continuous lines. Numbers refer to exons as shown in Fig. 3.

gene (codons CCT and CCC). The amino acids marking the end of the transin-hemopexin homology of Fig. 6A are encoded by exon 8 of the transin gene (data not shown). Thus the two similar halves of hemopexin appear to be distantly related to a duplication of the protein sequence encoded by exons 4 through 8 of the transin gene.

DISCUSSION

Transin RNA is produced in response to transformation of rat fibroblast cells by a number of oncogenes or by stimulation with EGF (32). We have shown that the ability to induce transin RNA is apparently a specific property of EGF, as neither PDGF, FGF, insulin, nor TGF-β is active in this respect in Rat-1 cells. The EGF induction of transin RNA can be blocked by the action of protein synthesis inhibitors, arguing that de novo synthesis of a protein or proteins is necessary for this response. What could these proteins be? EGF has been reported to increase the levels of c-*myc*, c-*fos*, and actin mRNAs in fibroblasts and other cell types (8, 15, 33, 37). The corresponding proteins are thus possible candi-

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GGATCCATCCCATATGTGGACACCAAATCCAGACACTGTTACTGATGCCAAGAAGTACTTGGTGACAGGAGCCTGATAGCTGTCTCCTGAGAGAATCTGCCAGAGCCTGACCAATACAGA
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TCCGGATGCTCACAGCCAACCATCAGACTGAGCACAGGGACCCCAAAAGAGGAGTTAGAAGGAAGACTGAAGAGTTAAAGGGTTTGCAACCCCATAGGAAGAACAACAATATCAACCAAC
                                                                                                                    360
CAGACCCCACTGAGATCCCAGGGACTAAATCACTAACCTAAGAGTACACATGTAGCAGAGGATGGCCTTATCTGGCATCAATGGGAAGGGAGGCCCTTGGTCCTATGAAGGCTCGATGCC
                                                                                                                    480
CCAGTGTATGAGAATGTTAGGGGCATGAGGTAGGAGTGAGTTGGTGAGTGGGAGAGCATCCTCATAGAACCAGGGGAGGGAGGACTGGATAGGGGGTTTGCAAAGGGAAAACCACAAAAG
                                                                                                                    600
CGGATAACATTTGAAATGTAAAAAAAAAAAAAAAAATAACCAATAAAAAATATCATAAAATAAGAAAGACAAATCTGACAGAAAAGATATAAGATAAAACCAGGCCATTCTACTTCAGTAA
                                                                                                                    720
ATCATATCAATTATATGAGCCTTTATAGAAAAAACTATTATAGACCCATCTCCTTTTAATATATGGGGTACAGAATGTGGGTCGTAGTGACAGACAGCGTGGGCTGTGGCTACAGAGGCA
                                                                                                                    840
CACCTTGTCCTTACCTCATCTCACTGTATTCAGCTTTGACTTCTGGAAGTTCTTTGTACAATTTGGACTTTTTACCAAGTTAGGCCACTACTATCCAAGTCATAAACATTACAGCTTCTG
                                                                                                                    960
AAGGATAGTTACATTTTCCAAAGTAGAAAAAAATGCCCCAGTTTTCTCTTTTGCTAAGGCAGGAAGCATTTCCTGGAGATTAATCACCATTCGCTTTGCAAAATTAAGAAGGTTTGAAGA
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ACTGAGTAAAGAAGATTATATCACTCTTCTGATTTTTAATTTTTGGAAATGGTCCCATTTGGATGGAAGCAATTATGAGTCAGTTTGCGGGTGACTCTGCAAATACTGCCACTCTATAAA
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AGTTGGGCTCAGAAAGGTGGACCTCGA
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FIG. 5. DNA sequence of the transin gene promoter region. The star indicates the mRNA cap site. CAAT and TATA box sequences are underlined. The 3′ end of the R family repeat sequence is indicated with an arrow.

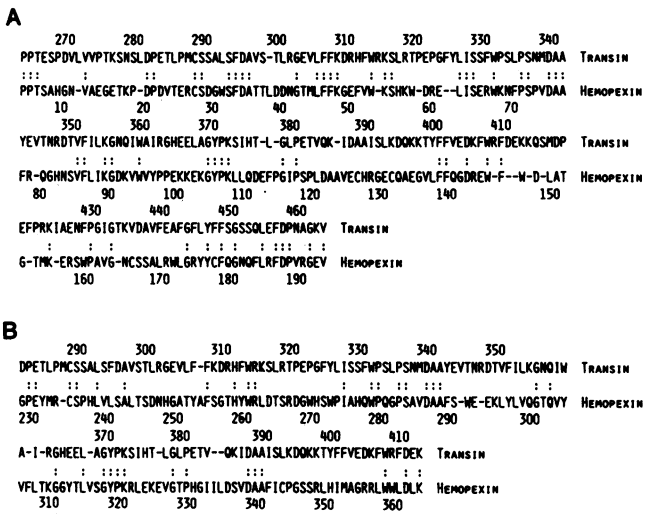

FIG. 6. Transin-hemopexin homologies. The transin sequence is from reference 32; the hemopexin sequence is from reference 55. Homologous residues are indicated by two dots. Sequence alignment was obtained using the computer program described by Wilbur and Lipman (61) with a gap penalty of 8.

dates. This effect of EGF is, however, shared by other growth factors, such as PDGF or FGF, and is a primary effect of the growth factor insofar as it is not blocked (but rather is reinforced) by protein synthesis inhibitors (10, 15, 25, 37). Egly et al. (14) have proposed a role for actin as a transcription factor, and *myc* and *fos* proteins are transported to the nucleus (6). It is tempting to speculate that these early, primary responses shared by several growth factors may act as instructions to the cell to get ready to transcribe, as a secondary response, a further set of genes whose expression would be necessary for the full response to the growth factor. Exactly which set of genes is chosen for transcription would depend on the particular growth factor used. In rat fibroblasts EGF would induce transin RNA, while another growth factor would be incapable of eliciting this response. The recent results of Frick et al. (17) show that in BALB/c-3T3 cells PDGF treatment leads to an increase (sensitive to cycloheximide) in levels of an mRNA encoding a 39-kilodalton lysosomal protein, whereas EGF treatment cannot elicit this response. (Transin RNA and the RNA described by Frick et al. are apparently different, as they encode proteins of different sizes [our unpublished data].) These secondary response genes would thus be growth factor specific, and perhaps cell type specific also, and may encode proteins with an important function in the animal in vivo where careful control of their expression must be achieved. It is interesting in this respect that transin RNA expression is under both positive and negative control in rat fibroblasts. Our results show that TGF-β can block the EGF induction of transin RNA. It is not clear at this time whether TGF-β acts directly on the transin gene or acts by blocking the EGF effect, possibly by reducing the number of high-affinity EGF receptors (30). TGF-β has recently been shown to be a bifunctional growth regulator. Thus, for example, it is known to inhibit the growth of AKR-2B and NRK rat fibroblasts in monolayer culture, while stimulating growth in soft agar of the same cells (42, 57). TGF-β can also block the peak of DNA synthesis produced by EGF and insulin 12 to 14 h after stimulation of AKR-2B cells (50). However, TGF-β is a potent stimulator of DNA synthesis in these same cells after a prolonged (≥24-h) prereplicative phase (50). Nevertheless, long-term (≥24-h) treatment of Rat-1 cells with TGF-β does not induce transin RNA, emphasizing the growth factor-specific nature of this response.

Both EGF treatment and transformation by various oncogenes of rat fibroblasts can lead to induction of transin RNA. In a search for a possible common mechanism of action of these factors, we have investigated the effect of some second messengers which have received attention as possible agents for transmitting the effects of oncogenes or growth factors. The interaction of many growth factors (e.g., PDGF, but perhaps not EGF) with their receptors leads to an activation of the phosphatidylinositol cycle, with production of second messengers which release intracellular calcium stores or activate protein kinase C (4). It has been suggested that the oncogenes *src* or *raf* might also play a role in this cycle (4). Furthermore, an early action of EGF is to effect a rise in intracellular calcium ion concentration (22). However, we have found that neither an increase of intracellular calcium ion concentration (effected by the calcium ionophore A23187) nor activation of protein kinase C by the phorbol ester TPA, nor a combination of the two, will result in induction of transin RNA in Rat-1 or FR3T3 cells. That activation of the phosphatidylinositol cycle alone is insufficient to induce transin RNA is consistent with the observation that PDGF will not induce transin RNA. However, activation of protein kinase C can play a role in control of transin RNA levels, as TPA treatment in the presence of serum will significantly enhance expression of transin RNA in Rat-1 cells. TPA will also induce transin RNA in mouse skin (L. M. Matrisian, G. T. Bowden, P. Kreig, G. Furstenberger, and R. Breathnach, manuscript in preparation) and the mouse epidermal cell line MCA3d (26).

The recent discovery that, in *Saccharomyces cerevisiae*, *ras* proteins may be controlling elements of adenylate cyclase (56) led us to investigate the effect of cAMP and cGMP on transin RNA levels in rat fibroblasts. In one cell line (FR3T3), dibutyryl cAMP treatments led to an increase in transin RNA levels, whereas in another (Rat-1) such treatments did not. Transformation of both lines by *ras* oncogenes leads to increased transin RNA levels, however, and recent evidence suggests that *ras* proteins are not linked to adenylate cyclase in higher eucaryotes (2). cAMP is thus not a likely candidate for a second messenger transmitting signals for EGF and oncogenes for transin gene transcription.

Is there a common meeting point for the mechanism of action of oncogenes, EGF, and TPA in induction of transin RNA? Our results showing that the microfilament-disrupting agent cytochalasin B can induce transin RNA suggest that this is indeed the case, and that the meeting point is the cytoskeleton, or more precisely the disruption of the cytoskeleton. Transformation by a variety of agents is known to disrupt microfilament organization and lead to loss of stress fibers and changes in cell shape (3). EGF (46) and TPA (41) also cause changes in cell shape, and in A431 cells EGF can effect a dramatic redistribution of actin fibers (46). Phosphorylation of adhesion plaque components such as vinculin or other cytoskeletal components such as p36 (3) by the tyrosine kinase activity of the EGF receptor (20, 23), $pp60^{v\text{-}src}$ (20, 47), or protein kinase C (60) could result in a disorganization of the adhesion plaque, loss of vinculin from the adhesion plaque, and an inability to link actin to the membrane and thus a loss of stress fibers. Disruption of microfilaments and their associated proteins in this way, or directly by the action of cytochalasin B, appears somehow to send a

signal to the nucleus leading to transcription of the transin gene. Our results reinforce the notion that cytoskeletal alterations are a meeting point between the actions of oncogenes and growth factors and may play a critical role in growth control.

Many of the genes induced by transformation or growth factor action and then isolated by differential hybridization contain repeated sequences or resemble retroviruses (16, 38, 52, 62). We undertook an analysis of the structure of the transin gene to determine whether this was also the case for this gene. Our results show that transin RNA does not contain repeated sequences and is probably encoded by a single-copy gene in the rat genome. The putative promoter element of this gene has classic CAAT and TATA boxes. No clear homologies between the promoter region of the transin gene and those of other genes (prolactin, c-*fos*, and VL30) induced by EGF could be detected. However, since EGF induction of transin RNA is not a direct effect, but depends on de novo protein synthesis, this latter gene may represent a class of secondary response genes whose control elements may not resemble those of primary response genes such as c-*fos* and might even not lie immediately adjacent to their cap sites. In this respect, it is interesting to note the close association of a LINE family element with the 5′ end of the transin gene and the presence of another within an intron of the gene (unpublished data); a role for repeated sequences in gene control has been the subject of much speculation.

We show here that the amino acid sequence encoded by the last four exons of the transin gene is distantly related to the two similar halves of the protein hemopexin, a plasma heme-binding protein (55). A similar situation holds for the low-density lipoprotein (LDL) receptor, one region of which is homologous to the EGF precursor protein and another is homologous to complement component C9 (53). It has been shown that a cassette of eight exons is shared by the genes for the LDL receptor and the EGF precursor (53, 54) and that much of the LDL receptor gene is made up of exons recruited from other genes by exon shuffling. Exon shuffling has also been observed in serine protease genes (43). It is tempting to speculate that exon shuffling has been used to share the protein coding sequence of exons 4 through 8 of the transin gene coding for the C-terminal moiety of transin with the hemopexin gene, though as the latter gene has not been isolated this hypothesis cannot at present be verified and other explanations for the homology are of course possible. Hemopexin binds heme and interacts with cell surface receptors on liver cells (55). Transin is a secreted protein (our unpublished data) and so might share either of these properties with hemopexin.

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