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Activation of the Ribosomal DNA Promoter in Cells Exposed to Insulinlike Growth Factor I

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We constructed a stable cell line, 3T3A5, which carried a chimeric gene in which the simian virus 40 T-antigen-coding gene was under the control of the mouse ribosomal DNA promoter. These cells expressed T antigen when they were growing exponentially in 10% fetal calf serum, but they all became T negative when incubated for 5 days in low-concentration serum. The readdition of serum or platelet-poor plasma again induced the expression of T antigen, which was accompanied by an increase in steady-state levels of the corresponding RNA. Among the various growth factors tested for their ability to induce T-antigen expression in 3T3A5 cells, only insulinlike growth factor I (IGF-I) could induce T antigen at physiological concentrations. The effect of IGF-I or platelet-poor plasma was abolished by an antibody to IGF-I. Other growth factors, like insulin and epidermal growth factor, could induce the expression of T antigen in 3T3A5 cells, but only at concentrations far above the physiological range. Other growth factors were totally ineffective. These results indicate that exposure of cells to IGF-I can activate transcription from the ribosomal DNA promoter.

Insulinlike growth factor I (IGF-I; somatomedin C) is a 70-amino-acid polypeptide closely related to insulin and IGF-II (3, 8, 19). The IGF-I gene is larger than 35 kilobase pairs (3) and maps to human chromosome 12 (6, 42). IGF-I is produced in the liver and many other adult and fetal tissues (43) and is present in the plasma of normal individuals at a concentration of 200 ng/ml. Its levels in plasma are the best indicator of growth in humans (8, 43). IGF-I plays a major role in the proliferation of fibroblasts in vitro (7). By itself, it cannot stimulate cellular DNA synthesis in quiescent BALB/c 3T3 cells, but it can do so if the quiescent cells have been previously exposed to nonmitogenic concentrations of platelet-derived growth factor (PDGF) (39, 43). In this respect, IGF-I (plus epidermal growth factor [EGF]) can replace platelet-poor plasma (PPP) as a progression factor in cells made competent by PDGF (22, 30; for a review, see reference 34).

It has been shown that progression but not induction of competence leads to an increase in cellular RNA content (35, 36). Since 85% of the cellular RNA is rRNA, we asked whether IGF-I could affect transcription from the ribosomal DNA (rDNA) promoter. For this purpose, we used a stable transformant of Swiss 3T3 cells developed in our laboratory, called 3T3A5 cells, which carry a chimeric gene in which the simian virus 40 (SV40) T-antigen-coding gene is under the control of the mouse rDNA promoter (40). The evidence that this chimeric gene is transcribed from the rDNA promoter is discussed in our previous paper (40) and below. The 3T3A5 cells are 100% T positive when grown in 10% fetal calf serum (FCS), but they all become T negative when incubated for a few days in low-concentration serum (see below). 3T3A5 cells, therefore, constitute an excellent tool for studying which growth factors can activate the rDNA promoter to express the T antigen.

MATERIALS AND METHODS

Cell lines. Swiss 3T3 cells and their derivatives, SV3T3 and Swiss 3T3A5, were maintained in culture, made quiescent, and stimulated as described previously by Kaczmarek et al. (20). The SV3T3 cell line was obtained by infection of Swiss 3T3 cells with SV40 and has been passaged in our laboratory for several years. Swiss 3T3A5 is the cell line established in our laboratory as described below in Results.

Growth factors. PDGF was a kind gift from J. Pledger. PDGF was dissolved in 0.1 M acetic acid at a concentration of 2 U/μml. PPP was prepared in our laboratory from fresh human blood, as described by Kaczmarek et al. (20). IGF-I (99% pure) was a gift from Am Gen Biologicals. IGF-I was dissolved in 0.1 M acetic acid at a concentration of 10 μg/ml. Transforming growth factor, a gift from M. Sporn, was dissolved in 4 mM HCl plus 1 mg of bovine serum albumin per ml at a concentration of 1 μg/ml. Insulin (92% pure; Sigma Chemical Co.) was dissolved in H_2O at a concentration of 10 μg/ml. Transferrin and EGF were purchased from Collaborative Research, Inc., and were dissolved as suggested by the manufacturer as stock solutions at concentrations of 1 mg/ml and 20 μg/ml, respectively. Growth factors were added to the cells contained within a cloning ring (diameter, 0.6 cm) either directly from stock solutions or after further dilutions in a conditioned medium.

Anti-IGF-I antibody. The anti-IGF-I antibody, a κIgG_1 monoclonal antibody purified from ascitic fluid, was obtained from J. J. Van Wyk. Its properties were described previously (33). Anti-IGF-I antibody was diluted in a conditioned medium and added to the cells as described above for growth factors.

Transfection and establishment of cell lines. Transfection and establishment of cell lines were performed as described before (40) (also see Results below).

Detection of SV40 large T antigen and [^{3}H]thymidine autoradiography. Cells were fixed 36 h after the addition of growth factor and were processed for [^{3}H]thymidine autoradiography or indirect immunofluorescence (31).

S1 mapping. The protocol used for S1 mapping was based on the one described by Maniatis et al. (25). Briefly, 10 μg of each RNA sample was hybridized for 3 h at 35°C to 10^5 cpm of 5'-end-labeled DNA probe. A 508-base-pair (bp) *Taq*I-*Taq*I fragment of pT (40) was used as the probe (see Fig. 6B). After hybridization, the samples were treated with 100 U of

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S1 nuclease (Bethesda Research Laboratories, Inc.) per ml for 20 min at 37°C. S1-resistant DNA-RNA hybrids were visualized by autoradiography after separation by denaturing electrophoresis on a 5% polyacrylamide gel containing 8 M urea (5).

RNA blots and nick translation. RNA blots and nick translation were carried out by standard procedures (32, 41).

Measurement of transcription rates in isolated nuclei. The runoff transcription assay was performed by combining several features of previously described techniques (10, 15, 17). Briefly, cells (10^8 per experiment) were washed with cold phosphate-buffered saline, trypsinized with 1% trypsin solution, neutralized with medium containing 10% FCS, collected by low-speed centrifugation, and suspended in lysis buffer (0.3 M sucrose, 2 mM magnesium acetate, 3 mM $CaCl_2$, 10 mM Tris [pH 7.5], 0.5% Nonidet P-40, 0.5 mM β-mercaptoethanol). Cells were then lysed by a brief Dounce homogenization, and nuclei were pelleted by centrifugation for 5 min at 1,000 rpm at 4°C. The nuclear pellet was washed once with 10 ml of reaction buffer (0.05 M $MgCl_2$, 0.01 M $MnCl_2$, 0.01 M Tris [pH 8], 0.14 M KCl, 1 mM dithiothreitol) and resuspended in 300 μl of the buffer described above containing 7.5% glycerol, 10 mmol each of ATP, CTP, and GTP per ml, and 500 Ci of [α-^{32}P]UTP (400 Ci/mmol; New England Nuclear Corp.). Reaction mixtures were incubated for 30 min at 30°C. Labeled nuclear RNA was isolated as described in detail by Engel et al. (10). Reaction mixtures were treated with 3 U of DNase I (Promega) in the presence of 20 μg of yeast tRNA at room temperature, 3 μl of 10-mg/ml proteinase K was added, and mixtures were incubated for 30 min at 42°C. The nucleic acids were then extracted with phenol and chloroform and precipitated with ethanol. After centrifugation (15,000 rpm, 30 min, 4°C), pellets of nucleic acids were dissolved in 300 μl of TE buffer (25) and passed through a Sephadex G-50 medium column. The proper fraction containing labeled RNA separated from free nucleotides was precipitated with ethanol, dissolved in 300 μl of DNase I buffer (20 mM HEPES [N-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.5], 5 mM $MgCl_2$, 1 mM $CaCl_2$), and incubated with 2 U of DNase I for 15 min at room temperature. The reaction mixtures were extracted with phenol and chloroform and used for hybridization. To detect labeled RNA transcribed from the chimeric gene, pMrT, pUC9, pSV2G, and phage DNAs were used. Each DNA sample was digested with *Hin*dIII restriction enzyme (Bethesda Research Laboratories) and bound to the nitrocellulose filter (7 μg of DNA per dot) by using dot blot apparatus (Schleicher & Schuell, Inc.) under the conditions recommended by the manufacturer. These filters were first prehybridized in 8 ml of solution containing 50% formamide, 2× Denhardt solution, 150 μg of salmon sperm DNA per ml, 5× SSPE, and 0.1% sodium dodecyl sulfate (25) for 2 h at 42°C. The filters were then hybridized for 5 days in the solution described above containing labeled RNA (~5×10^6 cpm/ml), washed twice with 2× SSC (1× SSC is 0.15 NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate at room temperature and three times with 0.2× SSC–0.1% sodium dodecyl sulfate at 55°C, and autoradiographed.

RESULTS

Establishment of cell lines. The chimeric gene used in these experiments, pMrT, has been described previously (40). It consists of a mouse rDNA promoter from the p5′ *Sal-Pvu* plasmid (26) and a promoterless T-antigen-coding gene from plasmid pSV2G (14) cloned in pUC9 (44). The mouse rDNA promoter includes 168 bp before and 57 bp after the transcription initiation site spliced to the *Bgl*I site (after blunt ending) of the SV40 genome. The SV40 fragment includes the cap site, splicing signals, and polyadenylation signal. When microinjected or transfected into mouse or hamster cells, the chimeric gene pMrT expresses T antigen (detectable by indirect immunofluorescence) and T-antigen mRNA; the gene is inactive when transfected into human or simian cells (40).

TABLE 1. T-positive cells among clone 3T3A5 and SV3T3 cells[a]

Cell line	% of T-positive cells			
	In exponential growth	In quiescent phase	With 10% serum[b]	With 10% serum + α-amanitin[b]
3T3A5	100	None	68	51
SV3T3	100	100	100	ND

[a] Cells were incubated in low-concentration serum (1.0% calf serum for 5 days). They were then exposed to 10% serum, either in the absence or presence of α-amanitin (2 μg/ml). The percentages of T-positive cells were determined by indirect immunofluorescence (30). ND, Not determined.
[b] Cells were fixed 36 h after the addition of serum.

Cell lines were established by cotransfection of Swiss 3T3 cells with pMrT and pSV2gpt (28), followed by selection for *gpt* as described previously (29, 40). Several of the selected clones were T positive, but the percentage of T-positive cells varied from one clone to another. In some clones, the percentage of T-positive cells was very low (~10%); in others, it was high (100%). The best clone, which was selected for these studies, was 3T3A5, in which 100% of exponentially growing cells were T positive (Table 1). When these cells were made quiescent by serum deprivation in 1% calf serum for 5 days, all the cells became T negative (Table 1 and Fig. 1). The readdition of serum (10% FCS) caused the reappearance of T antigen in 70% of the cells (Table 1 and Fig. 1). This clone appeared suitable for determining the ability of different growth factors to reactivate the rDNA promoter, especially since α-amanitin at concentrations that inhibit mRNA synthesis in cells in culture (27) had little effect on the percentage of T-positive cells induced by serum (Table 1).

Preliminary experiments indicated that the differences among clones were probably related to the number of integrated copies and the sites of integration. Relevant to the present study was the necessity to rule out the possibility of a position effect (23). We therefore investigated other cell lines carrying the integrated pMrT plasmid. Although the absolute numbers varied from one cell line to another, all 3T3-derived cell lines investigated (and one derived from Syrian hamster tsAF8 cells) behaved like clone 3T3A5; i.e., serum deprivation caused a marked reduction in or disappearance of T antigen, which reappeared when serum or PPP was added (data not shown). In contrast, in SV3T3 cells (Swiss 3T3 cells transformed by SV40, in which the SV40 T-antigen-coding gene is under the control of its natural, RNA polymerase II-directed promoter), the percentage of T-positive cells remained unchanged when the cells were transferred from high to low concentrations of serum (Table 1 and Fig. 1).

Dependence of the T-antigen-coding gene on the rDNA promoter. It is important to establish that the expression of T antigen (or its cognate RNA) by the pMrT plasmid depends on the presence of the rDNA promoter. The evidence that pMrT expression is dependent on the mouse rDNA promoter is partly given in this paper and was partly presented in the previous paper by Surmacz et al. (40). The

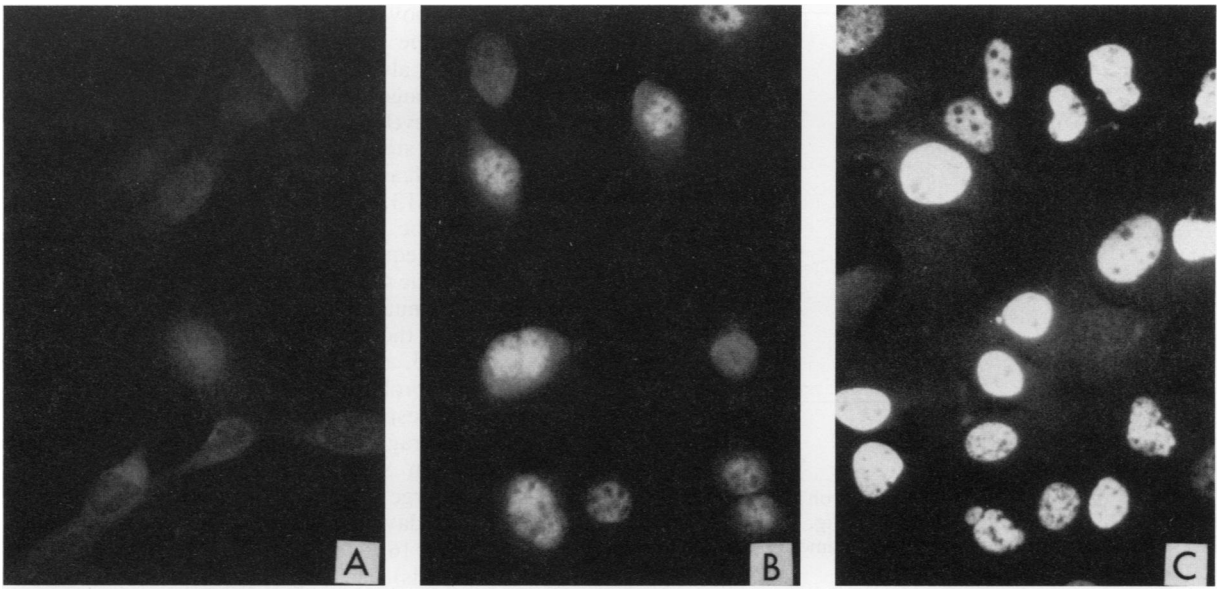

FIG. 1. Indirect immunofluorescence staining for SV40 T antigen. (A) 3T3A5 cells in low-concentration serum (1% calf serum). (B) 3T35A5 cells after the addition of 10% FCS. (C) SV3T3 cells in low-concentration serum.

evidence can be summarized as follows. (i) The expression of pMrT is sensitive to an antibody to RNA polymerase I (40) and is resistant to α-amanitin (Table 1). (ii) The promoterless T-antigen-coding sequence or a chimeric gene in which the rDNA promoter was cloned in the direction opposite to that in pMrT do not yield T-positive cells after transfection or microinjection (40). (iii) The expression of pMrT is species specific, as is expected from an rDNA promoter, while the expression of RNA polymerase II-directed transcripts is not species specific. (iv) Results from S1 nuclease assay (24) indicate that the majority of transcripts are correctly initiated at the transcription initiation site for RNA polymerase I (40), although some unusual transcripts were also detected, as already reported by Smale and Tjian (37). (v) Transcription from an integrated pMrT is not affected by the restrictive temperature in a mutant cell line which is temperature sensitive for RNA polymerase II activity (40).

The findings listed above support the conclusion that, in the integrated pMrT, the expression of T antigen is under the control of the rDNA promoter.

Effects of PPP and IGF-I on the percentage of T-positive cells. Figure 2 shows the effects of PPP and IGF-I on the expression of T antigen in 3T3A5 cells. The cells were made T negative with a low concentration of serum and then treated with different concentrations of either PPP or IGF-I. Both of these additions increased the percentage of T-positive cells. Note that the concentrations of IGF-I used are in the physiological range for plasma (43). While we realize that plasma IGF-I is largely bound to a carrier protein, it should be noted that the growth factors used in our experiments were added to a conditioned medium containing 10% serum. Figure 2 also shows the effects that PPP and IGF-I had on cellular DNA synthesis in quiescent Swiss 3T3 cells, the parent cell line of 3T3A5. Neither substance can induce cellular DNA synthesis, as is already well established in the literature (see reviews by Scher et al. [34] and also Kaczmarek et al. [20]). It should be mentioned that the DNA stimulation experiment cannot be done with 3T3A5 cells because the appearance of T antigen causes, by itself, the entry of cells into the S phase (DNA synthesis) (data not shown).

Figure 3 shows the effect that an antibody against IGF-I has on the percentage of T-positive cells induced by either PPP or IGF-I. In both cases, the addition of an antibody against IGF-I sharply reduced the percentage of T-positive cells. Indeed, the antibody was at least as effective with PPP as with IGF-I despite the fact that PPP was always more effective than IGF-I in rendering 3T3A5 cells T positive. The same antibody is known to inhibit the entry into the S phase of 3T3 cells which have been primed by PDGF and subsequently exposed to plasma (33).

Effects of PDGF and EGF. The same experiments (the addition of growth factors to quiescent 3T3A5 or Swiss 3T3 cells) were carried out with other growth factors. Figure 4

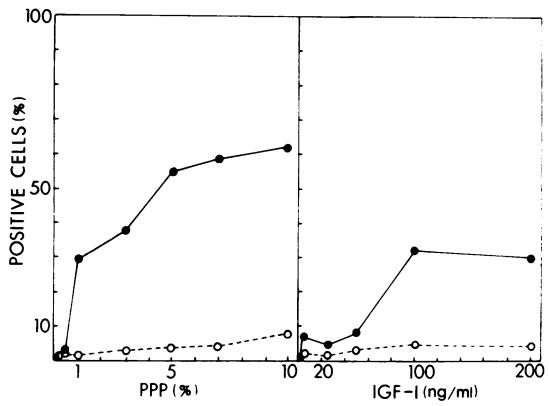

FIG. 2. Effects of PPP and IGF-I on the expression of T antigen in 3T3A5 cells. After 5 days in 1 % calf serum, the cells were treated with the concentrations of PPP or IGF-I shown on the abscissa. The percentage of T-positive cells (by indirect immunofluorescence) was scored 36 h later. In parallel experiments, quiescent Swiss 3T3 cells were treated with either PPP or IGF-I and then incubated with [^{3}H]thymidine for 36 h. The percentage of labeled cells was scored by autoradiography. Symbols: ●, percentage of T-positive 3T3A5 cells; ○, percentage of Swiss 3T3 cells labeled by [^{3}H]thymidine.

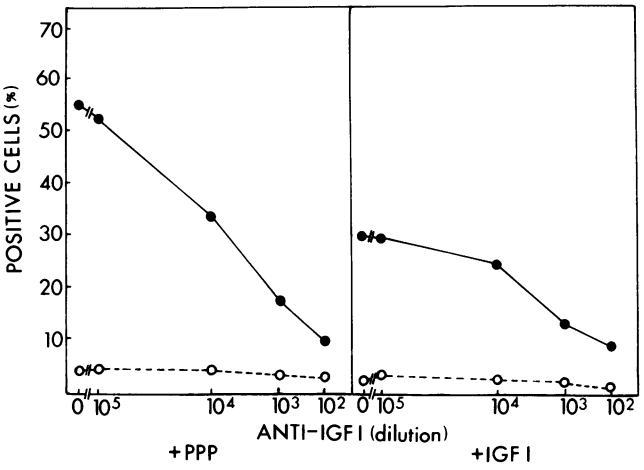

FIG. 3. Effect of an antibody against IGF-I on the percentage of T-positive 3T3A5 cells. Conditions are as for Fig. 2, except that the anti-IGF-I antibody was added at the same time as PPP (5%) or IGF-I (100 ng/ml). Symbols are as for Fig. 2.

shows the effects of PDGF and EGF. PDGF stimulated DNA synthesis in Swiss 3T3 cells, albeit weakly, and caused a very modest increase in the percentage of T-positive 3T3A5 cells, and this increase occurred only at concentrations at which DNA synthesis was stimulated. EGF had a very modest effect, in terms both of DNA synthesis in 3T3 cells and of percentage of T-positive cells in 3T3A5 cells. While 15% of the cells were T positive with 100 ng of EGF per ml, this concentration is well above the physiological range (<1 ng/ml, probably 300 pg/ml; G. Carpenter, personal communication).

Effects of other growth factors. Three other growth factors were tested, and the results are summarized in Fig. 5. Transferrin and transforming growth factor had no effect on the target cells, in terms either of DNA synthesis or of percentage of T-positive cells. Insulin, as expected, did not stimulate DNA synthesis in Swiss 3T3 cells but increased the percentage of T-positive cells in 3T3A5 cells. However, the concentrations of insulin used (100 ng/ml or more) are well above the physiological range of 0.3 to 0.6 ng/ml (18), and at these concentrations, insulin is known to interact with the IGF-I receptors (12, 21).

All these experiments were repeated several times, with reproducible results. Occasionally, insulin did not give any response with 3T3A5 cells; however, that result was always with an insulin preparation different from the source listed in Materials and Methods. With the preparations of growth factors listed above, the results were highly reproducible. For instance, the percentage of 3T3A5 cells rendered T positive by PPP always varied between 50 and 60%, while with IGF-I, it ranged from 30 to 40% (data not shown).

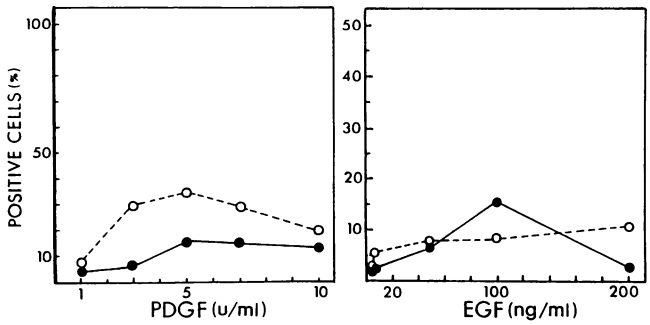

FIG. 4. Effects of PDGF and EGF on the expression of T antigen in 3T3A5 cells. Conditions and symbols are as for Fig. 2.

Steady-state levels of T-antigen RNA in 3T3A5 cells. We investigated the steady-state levels of cytoplasmic RNA by using an S1 nuclease assay. Samples of RNA isolated from 3T3A5, Swiss 3T3, and SV3T3 cells were hybridized to a radioactive probe (Fig. 6B) consisting of SV40 large T-antigen-coding sequences and SV40 intron sequences (from nucleotide residue 4722 to nucleotide 5218) and subsequently treated with S1 nuclease. Figure 6A shows our results from S1 analysis of the transcripts from SV3T3, Swiss 3T3, quiescent 3T3A5, and 3T3A5 cells stimulated by 5% PPP or 10% FCS. The probe used should protect a fragment of 333 nucleotides corresponding to the fragment of spliced large T mRNA. Such fragments were easily detectable when we used RNA from SV3T3 or stimulated 3T3A5 cells. The abundance of large T mRNA seemed to be similar in SV3T3 cells kept for 5 days in 1% calf serum and in the same cells restimulated for 16 h with 10% FCS (actually slightly lower in stimulated cells). The large T mRNA was not detectable in quiescent 3T3A5 cells or in the parental Swiss 3T3 cell line. Stimulation of 3T3A5 cells for 16 h caused T-antigen mRNA to appear very abundantly with 10% FCS or less abundantly with 5% PPP. It should be remembered that the activity of PPP on 3T3A5 cells was completely abolished by an antibody to IGF-I (Fig. 3). The weak band at 508 bp in all lanes of Fig. 6 corresponds to the size of reannealed probe. The same results were obtained with Northern blots (data not shown). Bands hybridizable to SV40 probes became detectable in RNA blots from 3T3A5 cells only after serum or PPP stimulation, while they were always present in RNA blots from SV3T3 cells, regardless of the concentration of the serum.

Run-off transcription assay. To determine more precisely whether the expression of the integrated pMrT was transcriptionally regulated, runoff assays were carried out on nuclei of 3T3A5 and SV3T3 cells under different conditions. The assay is described in Methods and Materials, and the results are shown in Fig. 7. Because of the large number of cells necessary for a runoff assay and the limited availability of IGF-I, we used only serum as a stimulant. However, serum is plasma plus PDGF, and we have shown (Fig. 4) that PDGF had a negligible effect on 3T3A5 cells. The effect shown in Fig. 7 can therefore be ascribed to the growth factors in the plasma.

The cells were stimulated for 6 h before the nuclei were prepared. Clearly, serum increased transcription of the pMrT gene in 3T3A5 cells, while it decreased the transcription of the SV40 T-antigen-coding gene driven by its natural promoter in Swiss 3T3 cells. Four growth-regulated genes, including c-*myc*, were also tested in 3T3A5 cells by runoff assay. Their transcriptional rates were not higher than in BALB/c 3T3 cells and were increased by the addition of serum (data not shown).

DISCUSSION

Cell line 3T3A5 is a stable transformant of Swiss 3T3 cells that carries the chimeric gene pMrT (40) in which the SV40 T-antigen-coding gene is driven by a mouse rDNA promoter (26). A similar, but not identical, construct was made by Grummt and collaborators (11, 16). Our construct had 57 bp between the initiation site of the mouse rDNA promoter and the beginning of the SV40 T-antigen-coding gene. In general,

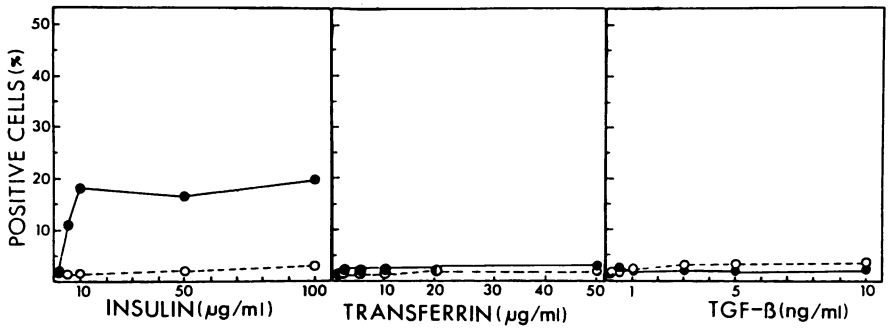

FIG. 5. Effects of other growth factors on the expression of T antigen in 3T3A5 cells. Conditions and symbols are as for Fig. 2. TGF-β, Transforming growth factor.

our results are in agreement with those of Grummt and collaborators (11, 16) in that the T-antigen-coding gene can be transcribed under the direction of an rDNA promoter, albeit at an efficiency lower than when the gene is under the control of its own promoter (see also Surmacz et al. [40]). The exposure of 3T3A5 cells to PPP or IGF-I resulted in an increased expression of T antigen controlled by the rDNA promoter. Since the activity of PPP was completely inhibited by an antibody against IGF-I (Fig. 3), it is reasonable to assume that the activity of PPP was largely due to IGF-I. However, PPP was more effective than IGF-I alone in stimulating T-antigen production, which suggests synergism of IGF-I with other growth factors. The exposure to PPP or serum also increased the levels of pMrT-directed RNA (Fig. 6), while it decreased the levels of T-antigen RNA transcribed from its own promoter. The experiments with SV3T3 (Table 1 and Fig. 5 and 6) served as controls to indicate that treatment with serum or PPP did not increase the stability of T-antigen mRNA or its protein. It would have been desirable to carry out the runoff transcription assay with IGF-I, but this assay was precluded by the limited availability of IGF-I and the great number of cells that were required. Still, the facts that PDGF was inactive and that the activity of PPP was abolished by an antibody to IGF-I justify the conclusion that the activation of the rDNA promoter is mediated by IGF-I. Since T antigen itself can activate the rDNA promoter (38), we chose it as a reporter to amplify the response. However, no T antigen would be available to stimulate the rDNA promoter in 3T3A5 cells in low-concentration serum unless the promoter itself was first activated. In addition, the choice of T antigen allowed us the use of SV3T3 cells as controls: if low-concentration serum destabilizes T antigen or its mRNA, it should do so also in SV3T3 cells, which, in fact, it did not(Table 1 and Fig. 6). Thus, even without the runoff transcription assay, our data indicate that serum, PPP, and IGF-I increase the synthesis of pMrT RNA.

No claim is made here that IGF-I acts directly on the rDNA promoter. On the contrary, since IGF-I is a growth factor, it probably interacts directly only with its own receptor. We can say, though, that the exposure of a cell to IGF-I resulted in the activation of the rDNA promoter. A more direct effect of IGF-I on the rDNA promoter could be demonstrated only by the use of cycloheximide to inhibit protein synthesis. Unfortunately, cycloheximide rapidly turns off any transcription from the rDNA promoter, a phenomenon that has been known for a long time (47) and which, incidentally, confirms that the rDNA promoter controls the transcription from pMrT.

Our experiments show that PPP induced the expression of

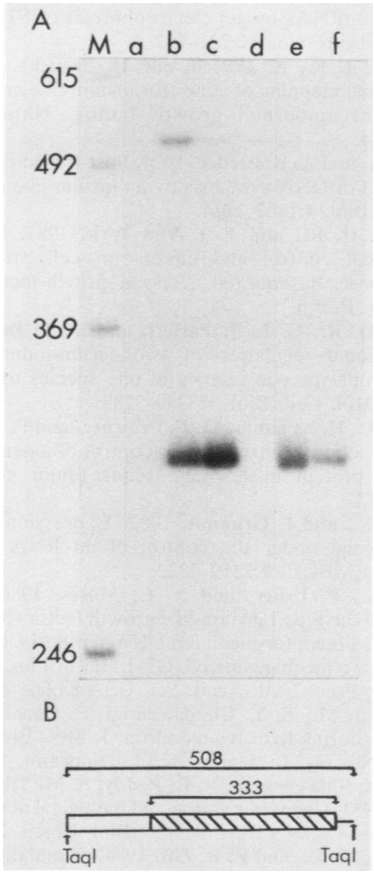

FIG. 6. S1 nuclease analysis of the steady-state cytoplasmic levels of large T mRNA in 3T3A5 and SV3T3 cells. The S1 nuclease assay was performed as described in Materials and Methods. (A) Autoradiograms of the S1-resistant fragments of hybrids between the probe and the following cytoplasmic RNAs. Lanes: a, total cytoplasmic RNA from 3T3A5 quiescent cells; b, total cytoplasmic RNA from 3T3A5 cells stimulated by 5% PPP for 16 h; c, total cytoplasmic RNA from 3T3A5 cells stimulated by 10% FCS for 16 h; d, total cytoplasmic RNA from Swiss 3T3 cells; e, total cytoplasmic RNA from SV3T3 cells kept for 5 days in 1% calf serum; f, total cytoplasmic RNA from SV3T3 cells restimulated with 10% FCS for 16 h; M, molecular weight markers. (B) Schematic drawing of the probe. The probe used contains SV40 large T-antigen coding sequences (▨) and SV40 intron sequences (□). The S1-resistant fragment of the RNA-DNA hybrid has a length of 333 nucleotides.

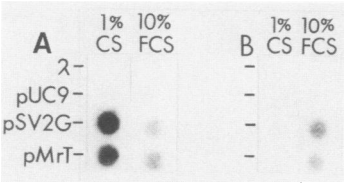

FIG. 7. Runoff transcription analysis of serum-deprived and restimulated 3T3A5 and SV3T3 cells. Nitrocellulose filters containing the indicated DNAs were hybridized to the runoff products (prepared as described in Materials and Methods) from SV3T3 (A) or 3T3A5 (B) cells that were serum deprived or restimulated by 10% FCS. Plasmids used in this experiment were pMrT (contains the large T-antigen gene driven by an rDNA promoter), pSV2G (contains the large T antigen under the control of its natural promoter), and pUC9. Phage DNA was also used as a negative control. CS, Calf serum.

T antigen and its RNA from the mouse rDNA promoter and so did IGF-I at physiological concentrations (200 ng/ml). Neither PPP nor IGF-I, under the same conditions, stimulated cellular DNA synthesis in quiescent Swiss 3T3 cells. The activation of the rDNA promoter by either PPP or IGF-I was inhibited by an antibody against IGF-I. It seems reasonable to conclude that IGF-I can mediate the activation of transcription from the rDNA promoter. It would be interesting to know whether IGF-I can activate other genes, especially RNA polymerase II-directed transcripts in the late G_1 phase (first gap phase). It should be noted, however, that according to Campisi and Pardee (7), IGF-I acts in the late G_1 phase on cell cycle progression in the presence of inhibitors of RNA polymerase II. It may be necessary, though, for the activation of the DNA-synthesizing machinery (46).

Other growth factors used in our experiments seemed to activate the rDNA promoter. However, both insulin and EGF were used at concentrations far above their physiological concentrations in plasma, which is 0.3 to 0.6 ng/ml for insulin (18) and 300 pg/ml for EGF (G. Carpenter, personal communication). It is generally agreed that, at superphysiological concentrations, insulin interacts with the receptors for IGF-1 (12, 21).

There has been for several years a lively controversy on whether the increased rRNA accumulation that certainly occurs in G_0-phase cells stimulated to proliferate is due to increased rRNA synthesis, decreased rRNA degradation, or both. Baserga (1) has discussed the various reports in a review, which also examined the pitfalls of some of the methods used. Runoff transcription should settle this controversy, but even here the reports are in apparent contradiction. Friedman et al. (13) reported no increase in rRNA transcription in regenerating liver after partial hepatectomy, but Edwards et al. (9) found an increase in rRNA transcription in G_0-phase fibroblasts stimulated by serum. In our cells and under the conditions we used, serum seemed to induce a higher rate of transcription from the rDNA promoter. Perhaps different mechanisms are operative in different types of cells.

Our results also indicate that transcription from an rDNA promoter is mediated by IGF-I. Since IGF-I, as stated above, plays a major role in cell cycle progression in the late G_1 phase, our results seem to confirm the suggestion of Adam and collaborators (35, 36) that rRNA accumulation is related to progression rather than competence. By no means should our results be construed as implying that IGF-I acts only on rRNA synthesis. Several DNA oncogenic viruses are known to stimulate cellular DNA synthesis (which requires unique-copy gene expression) and at the same time to cause an increase in rRNA synthesis. This is true of SV40 (38) and polyomavirus (4). IGF-I may act on the transcription of both rRNA and some unique-copy genes (but see the results of Campisi and Pardee [7] mentioned above). This is in fact possible, since IGF-I is necessary for the entry of 3T3 cells into the S phase (39, 43, 47), although rRNA synthesis is not strictly necessary for the same transition (2). It remains true, at any rate, that IGF-I can directly or indirectly activate transcription from the rDNA promoter, a finding that has significant implications in terms both of the biological effects of IGF-I and of the mechanism(s) by which the cell progresses through the cell cycle.

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LITERATURE CITED

1. **Baserga, R.** 1981. Introduction to cell growth: growth in size and DNA replication, p. 1–12. *In* R. Baserga (ed.), Tissue growth factors. Springer-Verlag KG, Berlin.
2. **Baserga, R.** 1984. Growth in size and cell DNA replication. Exp. Cell Res. **151**:1–5.
3. **Bell, G. I., D. S. Gerhard, N. M. Fong, R. Sanchez-Pescador, and L. B. Rall.** 1985. Isolation of the human insulin-like growth factor genes: insulin-like growth factor II and insulin genes are contiguous. Proc. Natl. Acad. Sci. USA **82**:6450–6454.
4. **Benjamin, T. L.** 1966. Virus-specific RNA in cells productively infected or transformed by polyoma virus. J. Mol. Biol. **16**:359–373.
5. **Berk, A. J., and P. A. Sharp.** 1977. Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease digested hybrids. Cell **12**:721–732.
6. **Brissenden, J. E., A. Ullrich, and U. Francke.** 1984. Human chromosomal mapping of genes for insulin-like growth factors I and II and epidermal growth factor. Nature (London) **310**:781–784.
7. **Campisi, J., and A. B. Pardee.** 1984. Post-transcriptional control of the onset of DNA synthesis by an insulin-like growth factor. Mol. Cell. Biol. **4**:1807–1814.
8. **Clemmons, D. R., and J. J. Van Wyk.** 1981. Somatomedin: physiological control and effects on cell proliferation, p. 161–208. *In* R. Baserga (ed.), Tissue growth factors. Springer-Verlag KG, Berlin.
9. **Edwards, D. R., C. L. J. Parfett, and D. T. Denhardt.** 1985. Transcriptional regulation of two serum-induced RNAs in mouse fibroblasts: equivalence of one species to B2 repetitive elements. Mol. Cell. Biol. **5**:3280–3288.
10. **Engel, D. A., H. Samanta, M. E. Brawner, and P. Lengyel.** 1985. Interferon action: transcriptional control of a gene specifying a 56,000-Da protein in Ehrlich ascites tumor cells. Virology **142**:389–397.
11. **Fleischer, S., and I. Grummt.** 1983. Expression of an mRNA encoding gene under the control of an RNA polymerase I promoter. EMBO J. **2**:2319–2322.
12. **Flier, J. S., P. Usher, and A. C. Moses.** 1986. Monoclonal antibody to the type I insulin-like growth factor (IGF-I) receptor blocks IGF-I receptor-mediated DNA synthesis. Clarification of the mitogenic mechanisms of IGF-I and insulin in human skin fibroblasts. Proc. Natl. Acad. Sci. USA **83**:664–668.
13. **Friedman, J. M., E. Y. Chung, and J. E. Darnell.** 1984. Gene expression during liver regeneration. J. Mol. Biol. **179**:37–53.
14. **Galanti, N., G. J. Jonak, K. L. Soprano, J. Floros, L. Kaczmarek, S. Weissman, V. B. Reddy, S. M. Tilghman, and R. Baserga.** 1981. Characterization and biological activity of cloned simian virus 40 DNA fragments. J. Biol. Chem. **256**:6469–6474.
15. **Greenberg, M. E., and E. B. Ziff.** 1984. Stimulation of 3T3 cells

induces transcription of the c-*fos* proto-oncogene. Nature (London) **311**:433–437.
16. **Grummt, I., and J. A. Skinner.** 1985. Efficient transcription of a protein-coding gene from the RNA polymerase I promoter in transfected cells. Proc. Natl. Acad. Sci. USA **82**:722–726.
17. **Hofer, E., and J. E. Darnell, Jr.** 1981. The primary transcriptional of the mouse β-major globin gene. Cell **23**:585–593.
18. **Jacobs, L. S.** 1980. Radioimmunoassay of peptide hormones, p. 435–468. *In* A. C. Sonnenwirth and L. Jarett (ed.), Gradwohl's clinical laboratory methods and diagnosis. The C. V. Mosby Co., St. Louis.
19. **Jansen, M., F. M. A. Van Shaik, A. T. Ricker, B. Bullock, D. E. Woods, K. H. Gabbay, A. L. Nussbaum, J. S. Sussenbach, and J. L. Van den Brande.** 1983. Sequence of cDNA encoding human insulin-like growth factor I precursor. Nature (London) **306**:609–611.
20. **Kaczmarek, L., J. K. Hyland, R. Watt, M. Rosenberg, and R. Baserga.** 1985. Microinjected c-myc as a competence factor. Science **228**:1313–1315.
21. **King, G. L., C. R. Kahn, M. M. Rechler, and S. P. Nissley.** 1980. Direct demonstration for separate receptors for growth and metabolic activities of insulin and multiplication-stimulating activity (an insulin-like growth factor) using antibodies to the insulin receptor. J. Clin. Invest. **66**:130–140.
22. **Leof, E. B., W. Wharton, J. J. Van Wyk, and W. J. Pledger.** 1982. Epidermal growth factor (EGF) and somatomedin C regulate G_1 progression in competent Balb/c 3T3 cells. Exp. Cell Res. **141**:107–115.
23. **Levis, R., T. Hazelrigg, and G. M. Rubin.** 1985. Effects of genomic position on the expression of transduced copies of the white gene of Drosophila. Science **229**:558–561.
24. **Lopata, M. A., B. Sollner-Webb, and D. W. Cleveland.** 1985. Surprising S1-resistant trimolecular hybrids: potential complication in intepretation of S1 mapping analysis. Mol. Cell. Biol. **5**:2842–2846.
25. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Miller, K., and B. Sollner-Webb.** 1981. Transcription of mouse rRNA genes by RNA polymerase I. Cell **27**:165–174.
27. **Monjardino, J. P.** 1978. The effect of α-amanitin on the synthesis of polyoma specific RNA. Biochem. Biophys. Res. Commun. **80**:1049–1058.
28. **Mulligan, R. C., and P. Berg.** 1980. Expression of a bacterial gene in mammalian cells. Science **209**:1422–1427.
29. **Mulligan, R. C., and P. Berg.** 1981. Selection for animal cells that express the *Escherichia coli* gene coding for xanthine-guanine phophoribosyltransferase. Proc. Natl. Acad. Sci. USA **78**:2072–2076.
30. **Pledger, W. J., C. D. Stiles, H. N. Antoniades, and C. D. Scher.** 1977. Induction of DNA synthesis in Balb/c 3T3 cells by serum components: reevaluation of the commitment process. Proc. Natl. Acad. Sci. USA **74**:4481–4485.
31. **Pope, J. H., and W. P. Rowe.** 1964. Detection of specific antigen in SV40 transformed cells by immunofluorescence. J. Exp. Med. **120**:121–130.
32. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity in vitro by nick-translation with DNA polymerase I. J. Mol. Biol. **113**:237–251.
33. **Russell, W. E., J. J. Van Wyk, and W. J. Pledger.** 1984. Inhibition of the mitogenic effects of plasma by a monoclonal antibody to somatomedin C. Proc. Natl. Acad. Sci. USA **81**:2389–2392.
34. **Scher, C. D., R. C. Shepard, H. N. Antoniades, and C. D. Stiles.** 1979. Platelet-derived growth factor and the regulation of the mammalian fibroblasts cell cycle. Biochim. Biophys. Acta **560**:217–241.
35. **Seuwen, K., and G. Adam.** 1983. Only one of the signals required for initiation of the cell cycle is associated with cellular accumulation of ribosomal RNA. Biochem. Biophys. Res. Commun. **117**:223–230.
36. **Seuwen, K., U. Steiner, and G. Adam.** 1984. Cellular content of ribosomal RNA in relation to the progression and competence signals governing proliferation of 3T3 and SV40-3T3 cells. Exp. Cell Res. **154**:10–23.
37. **Smale, S. T., and R. Tjian.** 1985. Transcription of herpes simplex virus *tk* sequences under the control of wild-type and mutant human RNA polymerase I promoters. Mol. Cell. Biol. **5**:352–362.
38. **Soprano, K. J., G. V. Devs, C. M. Croce, and R. Baserga.** 1979. Reactivation of silent rRNA genes by simian-virus 40 in human-mouse hybrid cells. Proc. Natl. Acad. Sci. USA **76**:3885–3889.
39. **Stiles, C. D., G. T. Capone, C. D. Scher, H. N. Antoniades, J. J. Van Wyk, and W. J. Pledger.** 1979. Dual control of cell growth by somatomedins and platelet-derived growth factor. Proc. Natl. Acad. Sci. USA **76**:1279–1283.
40. **Surmacz, E., Ø. Rønning, L. Kaczmarek, and R. Baserga.** 1986. Regulation of the expression of the SV40 T-antigen coding gene under the control of an rDNA promoter. J. Cell. Physiol. **127**:357–365.
41. **Thomas, P. S.** 1983. Hybridization of denatured RNA transferred on dotted nitrocellulose paper. Methods Enzymol. **100**:255–266.
42. **Tricoli, J. V., L. B. Rall, J. Scott, G. I. Bell, and T. B. Shows.** 1984. Localization of insulin-like growth factor genes to human chromosomes 11 and 12. Nature (London) **310**:784–786.
43. **Van Wyk, J. J., L. E. Underwood, A. J. D'Ercole, D. R. Clemmons, W. J. Pledger, W. R. Wharton, and E. B. Leof.** 1981. Role of somatomedin in cellular proliferation, p. 223–239. *In* M. Ritzer, K. Hall, A. Zetterberg, A. Aperia, A. Larsson, and R. Zetterstrom (ed.), The biology of normal human growth. Raven Press, New York.
44. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, and M13 mp-7 derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.
45. **Wilkinson, J., and B. Sollner-Webb.** 1982. Transcription of Xenopus ribosomal RNA genes by RNA polymerase I in vitro. J. Biol. Chem. **257**:14375–14383.
46. **Yang, H. C., and A. B. Pardee.** 1986. Insulin-like growth factor I regulation of transcription and replicating enzyme induction necessary for DNA synthesis. J. Cell. Physiol. **127**:410–416.
47. **Yu, F. L., and P. Feigelson.** 1972. The rapid turnover of RNA polymerase of rat liver nucleolus, and of its messenger RNA. Proc. Natl. Acad. Sci. USA **69**:2833–2837.