MOLECULAR AND CELLULAR BIOLOGY, Feb. 1993, p. 934–941
0270-7306/93/020934-08$02.00/0

Vol. 13, No. 2

In Vitro Transcription of *Drosophila* rRNA Genes Shows Stimulation by a Phorbol Ester and Serum

YESU CHAO AND MARIA PELLEGRINI*

Molecular Biology Program, University of Southern California, Los Angeles, California 90089-1340

Received 8 May 1992/Returned for modification 29 October 1992/Accepted 6 November 1992

The phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and serum both stimulate rapid increases in the transcription of *Drosophila* rRNA genes in vivo. Here we report that this stimulation is observed in in vitro transcription assays using nuclear extracts from cells treated with TPA or serum. Experiments in which extracts from TPA- or serum-treated cells were mixed with extracts from cells grown in serum-restricted medium showed that there was an increased RNA polymerase I (Pol I) activity present in the cell extracts from treated cells. We used a series of plasmids that had been deleted in the region 5′ to the start site of rRNA transcription to determine which sequences were necessary to support the increased transcription seen in extracts from stimulated cells. DNA templates that contain sequences between −150 and +32 (with +1 as the Pol I transcription start site) show dramatic increases in transcription with TPA- and serum-stimulated cell extracts; however, templates that contain 5′ sequences to −60 or −43 show at most one-third of the stimulation level of transcription in nuclear extracts from treated cells in comparison with untreated cell extracts. The 5′ deletion to −34 abolishes the stimulation effect and drops the basal-level transcription by 20-fold. These results indicate that the regulation of Pol I transcription in *Drosophila* cells by serum and TPA requires two DNA elements, sequences from −150 to −60 (upstream control element) and sequences from −43 to −34 (a portion of the core promoter).

The repeated genes coding for the large rRNAs in *Drosophila melanogaster*, as in other eukaryotic organisms, are transcribed by the RNA polymerase I (Pol I) system. This exclusive transcription of pre-rRNA by Pol I accounts for at least half of the total transcriptional activity in the cell. It has been demonstrated in several organisms that the rate of rDNA transcription is regulated with cellular growth rate (reviewed in reference 24). We have also shown that terminally differentiated, nongrowing secretory cells regulate ribosome production in response to the need for greater secretory protein synthesis (20). Tumor-promoting phorbol esters, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA), are known to effect the transcription of many RNA Pol II genes (reviewed in reference 9). We have found that TPA stimulates the Pol I transcriptional activity of *Drosophila* cells. This finding reflects the in vivo stimulation of rRNA transcription which we observed in nuclear run-on experiments (27). The rate of in vivo rDNA transcription is reflected in the activity (or lack of activity) of nuclear extracts (reviewed in reference 24). The changes reported for treatments such as serum starvation, amino acid starvation, and exposure of lymphosarcoma cells to glucocorticoids take place over relatively long times, hours to days (7, 8, 17).

To define regulatory events in the *Drosophila* system, we used Schneider Line-2 cultured cells, whose growth rate responds directly to the amount of serum in the culture medium. Here we report that shifting *Drosophila* cells from medium containing 2% fetal bovine serum to medium containing 15% serum stimulates the in vitro transcriptional activity of the Pol I system nearly ninefold within 1 h. Treatment of these cells with 5 nM TPA for only 15 min increases in vitro rDNA transcription 7- to 13-fold. We had shown previously that TPA, through a mechanism that involves protein kinase C, stimulates protein and RNA synthesis in *Drosophila* cells (28). Here we show that TPA can effect large changes in the Pol I transcriptional capacity of nuclear extracts prepared from these cells.

MATERIALS AND METHODS

Template-containing plasmids. The recombinant plasmid pDmr19 is a deletion to −150 bp at the 5′ end of pDmr275c2, which contains *Drosophila* rDNA. The −150 deletion of pDmr275c2 has the sequence from positions −150 to +680, which includes the gene promoter of rDNA and has +1 as the initiation site of rRNA. The recombinant plasmids pDmr146 and pDmr144 have upstream regions deleted to −60 and −43, respectively. The rDNA inserts cloned into pBR322 were kindly given to us by B. Kohorn (12). Template plasmids are diagrammed in Fig. 1. For measuring the activity of the gene promoter, several constructs were made by subcloning the rDNA inserts from the −150 deletion of pDmr275c2 into the vector pBluescript SK+ (Stratagene). pDmr608 contains a 608-bp *Hae*III-*Dra*I fragment that includes positions −150 to +441, pDmr199 contains a 199-bp *Hae*III-*Taq*I fragment that includes positions −150 to +32, and pDmr714 contains a 714-bp *Mnl*I-*Bam*HI fragment that includes positions −34 to +680. The overhanging ends of the inserts were filled in with the Klenow fragment and blunt-end ligated into the *Eco*RV site of pBluescript SK+ in a clockwise orientation toward the *Sac*I site on the vector.

Cell culture. Schneider Line-2 cells were routinely maintained in *Drosophila* Schneider's medium (GIBCO) supplemented with 15% fetal bovine serum (GIBCO), 0.1% (wt/vol) Yeastolate (Difco), 0.25% (wt/vol) Bacto Peptone (Difco), and 1% antibiotic-antimycotic (Sigma) (22).

Preparation of nuclear extract. Schneider Line-2 cells were grown in spinner flasks to mid-log phase (5×10^6 cells per ml). Extracts from 200 to 800 ml of cells were prepared by modification of a procedure described previously (16). The

* Corresponding author.

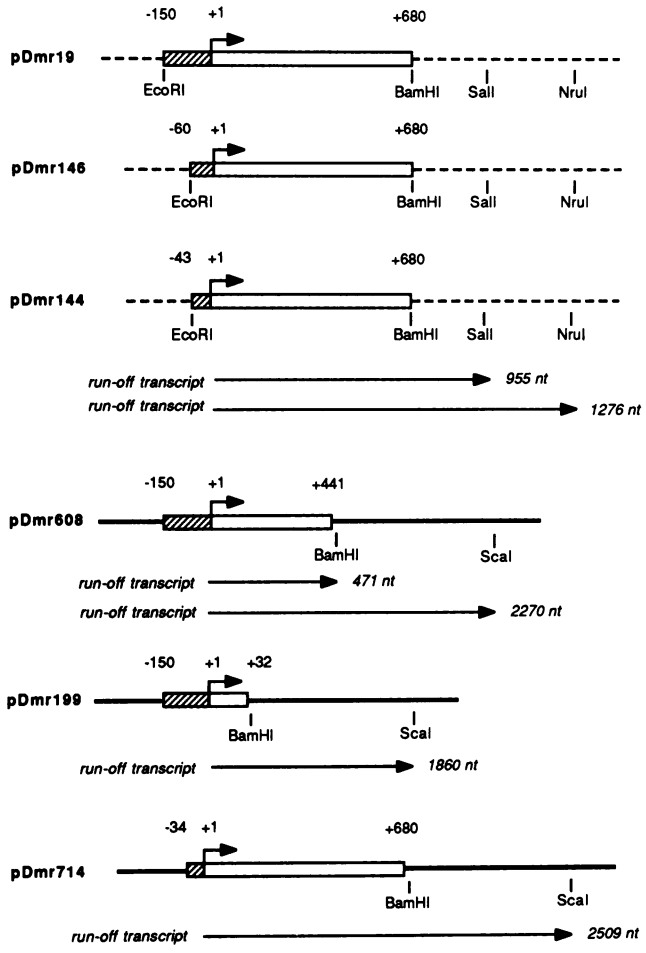

FIG. 1. Schematic diagram of DNA templates for in vitro transcription. pDmr19, pDmr146, and pDmr144 were constructed by subcloning 5′ deletion fragments of rDNA between −305 and +680 from pDmr275c2 into *Eco*RI and *Bam*HI sites of pBR322 (12). *Sal*I or *Nru*I is the restriction enzyme used to linearize these templates for runoff in vitro transcription. Sizes of the runoff transcripts are shown below these DNA templates. pDmr608, pDmr199, and pDmr714 were constructed by subcloning the rDNA fragment from pDmr19 into the *Eco*RV site of pBluescript SK+ (see Materials and Methods). *Sca*I or *Bam*HI was used to linearize these templates for runoff in vitro transcription. The size of the runoff transcript is shown below each template. +1 is the initiation site for transcription. Symbols: striped box, upstream promoter region of rDNA; open box, coding region of rDNA; dotted line, pBR322; black line, pBluescript SK+; arrow at +1, direction of transcription.

cells were washed in 10 volumes of buffer A (15 mM KCl, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], 2 mM $MgCl_2$, 0.1 mM EDTA). Before homogenization, the cellular pellet was resuspended in 10 ml of buffer A supplemented with 1 mM dithiothreitol (DTT) per liter of cells and put on ice for 15 min. After centrifugation, the nuclear pellet was resuspended in 9 parts buffer A and 1 part buffer B (1.0 M KCl, 50 mM HEPES [pH 7.9], 30 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT). The protein was precipitated by the addition of 0.35 g of ammonium sulfate per ml of supernatant. The protein pellet was redissolved in 1 ml of buffer C (20% glycerol, 100 mM KCl, 20 mM HEPES [pH 7.9], 0.2 mM EDTA, 0.5 mM DTT) per 10^9 cells and dialyzed against buffer C for at least 4 h.

Runoff in vitro transcription reactions. A standard transcription reaction mixture was 25 μl and contained (final concentrations) 9.6 mM HEPES (pH 7.9), 48 mM KCl, 0.1 mM EDTA, 9.6% glycerol, 0.24 mM DTT, 10 mM $MgCl_2$, 600 μM ATP, 600 μM CTP, 600 μM UTP, 40 μM GTP, 0.5 μM (10 μCi) [α-^{32}P]GTP (800 Ci/mmol), α-amanitin at 0.3 mg/ml, 20 U of RNasin (Promega), and a linear double-stranded DNA template at 8 to 40 μg/ml. Reactions were initiated by addition of nuclear extract in the amounts indicated in the figure legends. Mixtures were incubated at 25°C for 4 min. Reactions were then terminated by addition of 1% sodium dodecyl sulfate, 100 mM NaCl, 100 mM Tris (pH 8.0), 10 mM EDTA, and a final concentration of 0.5 mg of carrier tRNA per ml. The RNA was purified by phenol extraction and ethanol precipitation and electrophoretically resolved on a 1.5% aragose gel after denaturation with glyoxal and dimethyl sulfoxide (19). The in vitro transcripts were visualized by autoradiography. Relative levels of transcription were determined by densitometric scanning of autoradiographs from four to five independent reactions, using an Ultrascan XL laser densitometer (LKB).

S1 nuclease mapping. S1 nuclease protection assays were carried out according to standard techniques (19). In Fig. 4, the end-labeled DNA probe was prepared from plasmid pDmr199. The plasmid was cleaved with *Bam*HI and labeled on the template strand with [γ-^{32}P]ATP and polynucleotide kinase. The labeled DNA fragment was then cleaved with *Kpn*I. The fragment containing the rDNA sequences from −150 to +32 was gel purified. The double-stranded DNA probe was denatured and electrophoresed on a 4% sequencing gel, from which single-stranded DNA probe was prepared and isolated. In Fig. 6, the DNA probe was prepared from pDmr144. The plasmid was cleaved with *Rsa*I, and the fragment containing rDNA −43 to +95 was isolated. The single-stranded DNA probe was prepared by digestion with exonuclease III (19). Excess single-stranded DNA probe and DNase I-treated in vitro transcription reaction products were incubated for 12 to 16 h at 45°C (Fig. 4) or 30°C (Fig. 6) in 40 μl of 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.4)–1 mM EDTA–0.4 M NaCl–80% formamide. Following S1 nuclease digestion, protected DNA fragments (Fig. 4) or RNA fragments (Fig. 6) were analyzed on denaturing polyacrylamide gels (19). The corresponding end-labeled, double-stranded DNA fragment was cleaved by base-specific chemical reactions (14) and electrophoresed on the same gel.

RESULTS

In vitro transcription systems for *Drosophila* Pol I. To investigate the regulation of rDNA transcription in *Drosophila* cells, we first tested the original rDNA transcription system developed by Kohorn and Rae (10). A plasmid DNA which contained rDNA sequences from −150 to +680 (where +1 is the start site of transcription) cloned in pBR322 was used as the template. When restricted with *Sal*I, it gives a runoff transcript of 955 nucleotides (nt). We found that in our hands, the S-100 (mostly cytoplasmic) extracts made according to Kohorn and Rae (10) gave transcripts of 955 nt which showed optimal reaction times of 4 to 5 min and α-amanitin insensitivity. More recently, Parker and Topol (16) reported a protocol for producing transcriptionally active nuclear extracts from *Drosophila* cells. We have used this method to test the activity of Pol I transcription on the same rDNA template. A 955-nt transcript was again produced within an optimal reaction time of 4 min and was

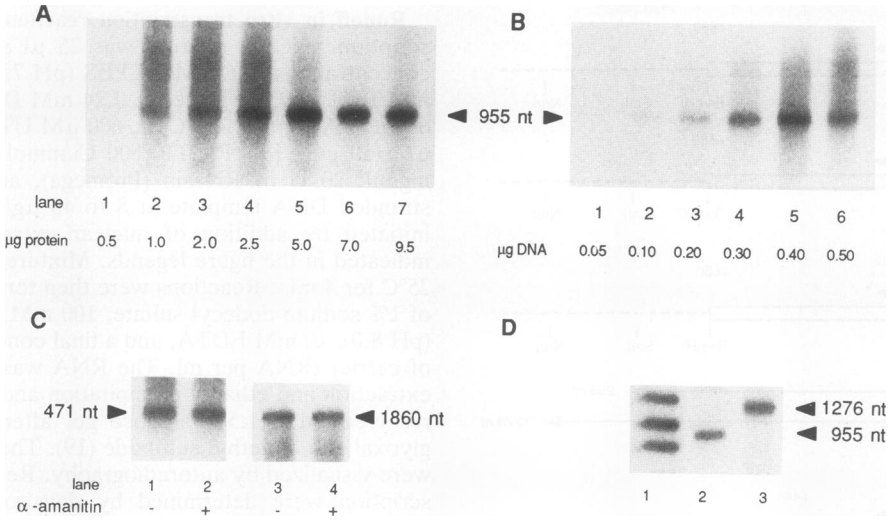

FIG. 2. (A) Optimization of in vitro transcription by varying the amount of nuclear extract. *Sal*I-digested pDmr19 was used as the template for runoff transcription. Each reaction contained 0.28 μg of DNA template. Increasing amounts of extract from cells grown in 15% serum were added to 25 μl of transcription mixture. RNA was purified, run on a 1.5% agarose gel after denaturation with glyoxal and dimethyl sulfoxide, and visualized by autoradiography. (B) Optimization of in vitro transcription by varying the amount of DNA template. Increasing amounts of *Sal*I-digested pDmr19 were used as templates for runoff transcription. For each lane, 5 μg of extract from cells grown in 15% serum was added. Purified RNA was detected as for panel A. (C) α-Amanitin insensitivity of in vitro transcription of rDNA sequences. Two rDNA templates are represented: *Bam*HI-digested pDmr608 (lanes 1 and 2) and *Sca*I-digested pDmr199 (lanes 3 and 4). The final concentration (300 μg/ml) of α-amanitin was present (+) or absent (−) in the reaction. For each lane, 7 μg of extract from cells grown in 15% serum was added. (D) Evidence that in vitro transcription of rDNA sequences is independent of surrounding vector DNA. pDmr19 was cut with *Sal*I (lane 2) or *Nru*I (lane 3). Each reaction contained 0.4 μg of DNA template and 5 μg of extract from cells grown in 15% serum. Lane 1, size marker (^{32}P-end-labeled ϕX174 digested with *Hae*III).

insensitive to an α-amanitin concentration of 300 μg/ml. These results indicate that transcription was effected by the Pol I system.

Having demonstrated that transcription of the rDNA template was directed by the Pol I system, we optimized this transcription system for template concentration (0.4 μg of DNA) and for the protein concentration of the extract (5 μg) (Fig. 2A and B). This amount of protein is far less than that required for transcription with the S-100 extracts (>20 μg). Because of the higher activity of the nuclear extracts, we have used them for all of our further studies. A comparison of transcription with and without α-amanitin (300 μg/ml) for two different templates is shown in Fig. 2C. The equivalent levels of transcription seen in the presence and absence of this inhibitor indicate that transcription is mediated by Pol I, regardless of whether the rDNA sequences extend from −150 to +441 or only to +32 (Fig. 2C).

Using different restriction enzymes, we produced templates that had identical rDNA sequences but gave different-size runoff transcripts. As seen in Fig. 2D (lanes 2 and 3), both templates gave transcripts of the expected sizes, indicating that there is little effect of the surrounding plasmid sequences on transcription.

Stimulation increases in vitro transcriptional activity. Bovine serum is required for the growth and propagation of Schneider Line-2 tissue culture cells (22). As detailed in the accompanying report (27), these cells grow rapidly (doubling every 24 h) when maintained in medium containing 15% serum and grow slowly (doubling every 50 h) when maintained in medium containing 2% serum. When slowly growing cells are stimulated by the addition of medium containing 15% serum, they show increased protein synthesis and increased transcription of rDNA (27). We have investigated the transcriptional activity of nuclear extracts prepared from both slowly growing (on 2% serum) and rapidly growing (on 15% serum) *Drosophila* cells. The template used in the reactions contains rDNA from −150 to +680 and includes all of the sequences that were determined by Kohorn and Rae (11) to be necessary and sufficient for accurate and efficient Pol I transcription. The results of in vitro transcription experiments are shown in Fig. 3. We also assayed the level of transcriptional activity present in extracts from cells that had been grown in 2% serum and were stimulated by the addition of serum to final concentration of 15% for either 1 or 3 h. Figure 3 shows the level of transcription with use of an equal amount of protein from each extract. Rapidly growing

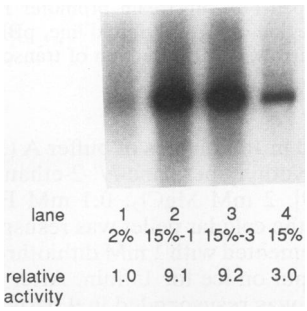

FIG. 3. Stimulatory effect of serum on RNA Pol I transcription. In the runoff transcriptions, 0.4 μg of *Sal*I-digested pDmr19 was used in each reaction. Equal amounts of nuclear extracts were added, and the rDNA transcripts were analyzed as for Fig. 2. Relative levels of transcription were determined by densitometric scanning of autoradiographs. Levels of increase compared with a value of 1.0 for the activity of nuclear extracts prepared from cells growing in 2% serum are shown at the bottom.

cells (lane 4) show a threefold-higher level of transcription than do slowly growing cells. One hour following serum stimulation, nuclear extracts supported transcription at a level ninefold higher than that observed in extracts from cells grown continuously with 2% serum. Three hours after serum treatment, nuclear extracts from the cells also showed transcriptional activity approximately ninefold higher than that from slowly growing cell extracts, which exceeded the level seen in cells maintained in 15% serum. This result was similar to that which we obtained in nuclear run-on assays; i.e., the level of rDNA transcription observed in vivo for serum-stimulated cells was even greater than that from cells maintained in high serum (27).

To determine whether there was any increased activity that could cause our in vitro transcripts to be more rapidly degraded in extracts of slowly growing cells, we incubated randomly labeled rRNA made from a T7 phage promoter in each of our extracts. Reactions contained all of the components present in our in vitro transcription reactions, including all four nucleoside triphosphates (none was radioactive). Following the standard 4-min incubation, the T7-transcribed rRNA was isolated and assayed by gel electrophoresis. No detectable degradation or change in size of this rRNA was observed (data not shown).

Exposure of cells to TPA also increases transcriptional activity. The phorbol ester TPA is known to stimulate the activity of protein kinase C. We had previously observed that TPA stimulated protein synthesis in the terminally differentiated male accessory gland cells of *D. melanogaster* and that TPA caused increases in rDNA transcription, as judged by nuclear run-on assays (27). We have now determined that this increased in vivo transcription is reproduced in extracts from TPA-treated cells. To determine that the accurate initiation is not altered by serum and TPA treatments, the initiation site of transcription was examined by S1 nuclease mapping. Figure 4 demonstrates that the in vitro transcripts produced in this system have the correct 5′ start site as determined by Kohorn and Rae (10, 12). These results also show that in stimulated cell extracts, the start site of transcription is not altered although the amount of transcription changes. In addition, Fig. 4 shows that the extract prepared from TPA-treated cells supports a similar increase in transcriptional activity compared with extract from serum-stimulated cells.

To determine the *cis*-acting sequences that may mediate the increase in transcription we observed, we studied a series of deletions around the start site of transcription. Figure 5 shows the relative transcriptional activities of nuclear extracts derived from control cells (grown with 2% serum), cells stimulated with serum or TPA, and those grown continuously with 15% serum. Figure 5A shows the results of transcription experiments using these four extracts and the −150 to +680 template. This is the template (Fig. 3) that supports an 8- to 11-fold increase in transcriptional activity by serum and TPA treatment.

We next examined the influence of 3′ sequences on the ability of the rDNA template to support increased transcription in extracts derived from serum or TPA-stimulated cells. A template containing rDNA from −150 to +441 (deleting the sequences from +442 to +680) continued to support regulated transcription (Fig. 5B and C). We had previously found that sequences from +558 to +580 contained 65% identity with the consensus sequence for the serum response element (SRE) in mammalian cells (15). This construct eliminated those potential regulatory sequences yet still showed stimulated transcription in serum-treated cell extracts. Then, a plasmid containing rDNA sequences from −150 to +32 was tested. It is clear that this construct also supports transcription of rDNA and is regulated to the same degree as is the −150 to +680 template when extracts from stimulated cells are used (Fig. 5D). Therefore, the sequences downstream of +32 do not appear to play a role in the regulation of rDNA transcription by serum or TPA.

To examine whether vector DNA has an effect on the stimulation, the template containing rDNA from −150 to

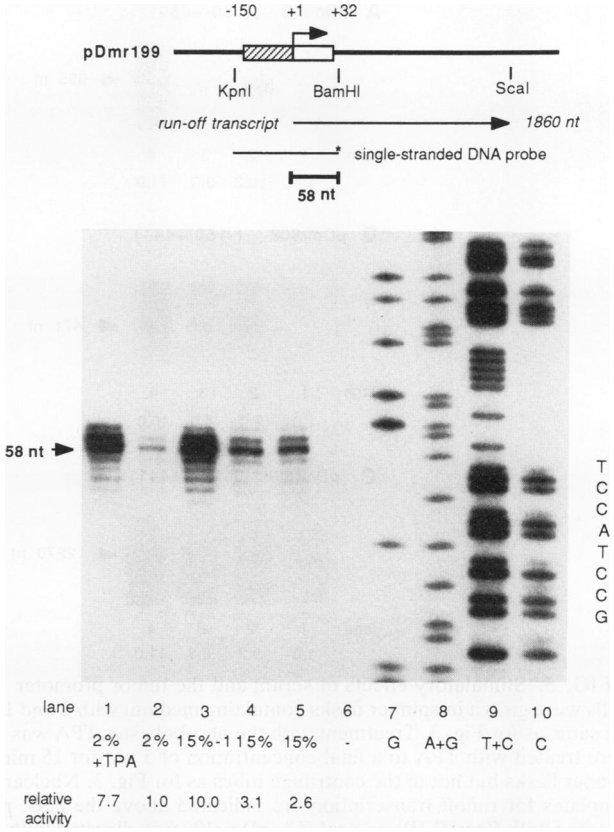

FIG. 4. Nuclease S1 mapping with DNA templates containing rDNA from −150 to +32. A schematic diagram of the single-stranded DNA probe, runoff transcript, and 58-nt protected DNA fragment is shown at the top. The DNA probe was a *Bam*HI-*Kpn*I segment 5′ end labeled at the *Bam*HI site. End-labeled noncoding strand (template strand) was purified from polyacrylamide gels (see Materials and Methods). In the runoff in vitro transcription reactions, 0.4 μg of DNA template pDmr199 linearized with *Sca*I was added in each reaction, and 7 μg of nuclear extract was present (lanes 1 to 5) or absent (lane 6). The type of extract used is indicated under each lane. α-Amanatin (300 μg/ml) was present (lanes 1 to 4) or omitted (lane 5). Following the in vitro transcription reaction, DNA templates were removed by DNase I digestion. RNA transcripts were then hybridized with excess DNA probe and treated with S1 nuclease. The protected DNA fragments were analyzed on 10% denaturing polyacrylamide gels. The double-stranded DNA probe was chemically sequenced (lanes 7 to 10). The protected DNA fragment runs, as expected, 1.5 nt lower than its chemically modified homolog because the sequencing reaction eliminates the modified nucleotide and leaves a 3′ phosphate group. The arrow points to the band that was prominent as a result of the preferential digestion of nuclease S1 at the 5′ triphosphate of the RNA transcript. Relative levels of transcription were determined by PhosphorImager (Molecular Dynamics) analysis. Levels of increase compared with a value of 1.0 for the activity of nuclear extracts prepared from cells growing in 2% serum are shown at the bottom.

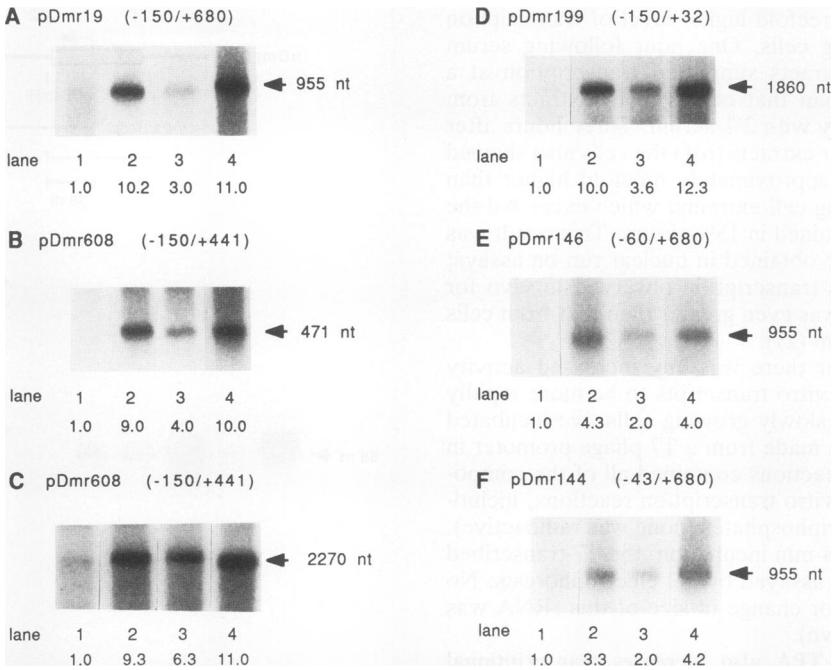

FIG. 5. Stimulatory effects of serum and the tumor promoter TPA on RNA Pol I transcription of different 5′ and 3′ deletion templates. Cells were grown in spinner flasks containing medium with 2 and 15% serum, respectively. Cell growth conditions and serum treatment were the same as for Fig. 3. Treatment with the phorbol ester TPA was done when the cells grown in 2% serum reach the mid-log phase. The cells were treated with TPA to a final concentration of 5 nM for 15 min. During treatment with serum and TPA, the cells were still grown in the spinner flasks but not in the centrifuge tubes as for Fig. 3. Nuclear extracts were prepared as described in Materials and Methods. The DNA templates for runoff transcription are indicated above the gels. pDmr19, pDmr146, and pDmr144 were digested with *Sal*I. pDmr608 was digested with *Bam*HI (B) or *Sca*I (C). pDmr199 was digested with *Sca*I. Each transcription reaction contained 0.4 μg of DNA template. The schematic diagram of DNA templates and the sizes of transcripts are depicted in Fig. 1. The reaction was initiated by addition of nuclear extract from cells grown in 2% serum (lanes 1), cells shifted to 15% serum for 1 h (lanes 2), cells grown in 15% serum (lanes 3), or cells treated with TPA for 15 min (lanes 4). In panel A, 7 μg of protein was added; in panels B to D, 8 μg of protein was added; in panels E and F, 9 μg of protein was added. The rRNA transcripts were analyzed as for Fig. 2. Relative levels of transcription were determined as described in the legend to Fig. 3.

+441 was cut with either *Bam*HI or *Sca*I. These templates should yield different-size RNA transcripts of 471 and 2,270 nt, respectively. Both of these templates showed stimulated transcriptional activity, indicating that vector DNA does not contribute to the stimulatory effect (Fig. 5B and C).

We next investigated the ability of different 5′ sequence elements in the rDNA template to support increased in vitro transcription in the serum- and TPA-stimulated extracts. Templates that contained deletions of 5′ sequences to −60 and −43 with 3′ rDNA to +680 were transcribed in our in vitro system. These constructs contained the 5′ core, minimal sequences which Kohorn and Rae (11, 12) reported could support transcription in vitro. As shown in Fig. 5E and 5F, each of these templates showed increased transcriptional activity in extracts from serum- or TPA-treated cells. However, the increase in transcription was less than the increase observed for a template containing 5′ sequence to −150. Our assays showed that the −60 and −43 templates supported no more than a 3.5-fold stimulation in transcriptional activity in extracts from serum-treated cells, compared with a 9-fold increase observed with the −150 template in the same extract. Similarly, the −60 and −43 templates showed an average 4-fold increase in transcriptional activity in extracts from TPA-treated cells, while the −150 template supported a 10-fold increase. The results of several experiments using these templates are summarized in Table 1.

Third, we tested a plasmid that contained 5′ sequences deleted to −34. This template gave barely detectable levels of transcript, whichever extract was used (data not shown). This finding confirms the results of Kohorn and Rae that sequences 5′ of −34 are required for basal-level rDNA transcription. It also shows that this template cannot support increased transcription in extracts that do show stimulation on templates containing sequences between −150 and +32. S1 nuclease mapping of the in vitro transcripts from the −34 template shows that it supports correct initiation and con-

TABLE 1. Stimulation of Pol I transcriptional activity by serum and TPA[a]

5′ deletion	Activity (mean ± SE)[a] after treatment with:	
	Serum (1 h)	TPA
−150[b]	9.43 ± 1.58	11.06 ± 1.70
−60[b]	3.12 ± 1.15	3.54 ± 1.27
−43[b]	3.14 ± 0.13	4.41 ± 1.29
−34[c]	1.22 ± 0.01	0.99 ± 0.07

[a] Relative to a value of 1.0 for the activity of each separate template in nuclear extracts from cells growing on 2% serum.
[b] Data are from at least four independent experiments.
[c] Data are from two independent experiments. The control value of the transcriptional activity from this template is 20-fold lower than that for the other templates.

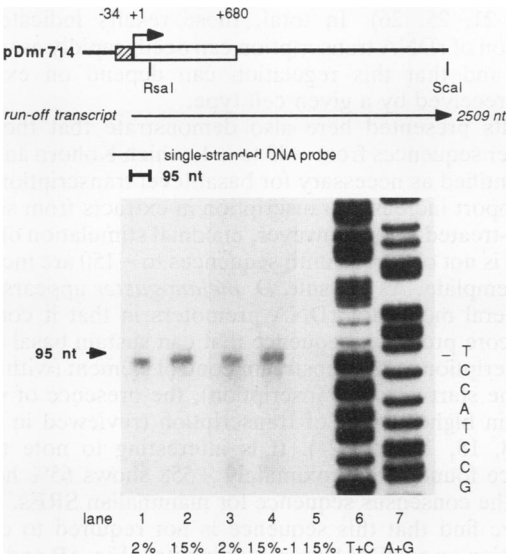

FIG. 6. Nuclease S1 mapping with DNA templates containing rDNA from −34 to +680. A schematic diagram of the single-stranded nonradioactive DNA probe, runoff transcript (radioactive), and 95-nt protected RNA is shown at the top. The single-stranded DNA probe was prepared by exonuclease III digestion (19) and has the 5′ end at +95. In the runoff in vitro transcription reactions, 0.4 μg of DNA template pDmr714 linearized with *Sca*I was added (lanes 1 to 4) or omitted (lane 5), and 7 μg of nuclear extract was present in the reaction. The type of extract used is indicated under each lane. The reactions of hybridization and nuclease S1 digestion were the same as for Fig. 4 except that the hybridization temperature was 30°C. For chemical sequencing, the double-stranded DNA probe was 5′ end labeled at +95; the sequences are shown in lanes 6 and 7. The protected RNA fragments were analyzed on 6% denaturing polyacrylamide gels. The 5′ ends of the in vitro-transcribed RNAs contain three phosphates; thus, the chemically modified DNA in the sequencing lanes migrates 0.5 nt faster.

firms that the levels of transcripts produced are similar in all of the extracts (Fig. 6).

The increases in transcription are due to stimulatory activity present in serum and TPA-treated extracts. To determine whether the increased transcription that we observed in extracts from serum-stimulated and TPA-treated cells was due to an increase of a stimulatory activity or to the removal of a transcriptional inhibitor, we performed a series of mixing experiments. In each experiment, the total protein concentration of extract was maintained at a constant amount. Extract prepared from slowly growing cells (growing in 2% serum) was mixed with extracts from cells stimulated for 1 h with 15% serum, maintained in 15% serum, or stimulated with TPA (Fig. 7A). The template used in these experiments contains rDNA sequences from −150 to +680. Clearly, the addition of extract from stimulated cells increases the transcriptional activity of the extracts prepared from slowly growing cells. The amount of increased transcriptional activity shown in the mixed extracts is similar in magnitude to that seen in the stimulated cell extracts by themselves. That is, the greatest activity is found in the TPA-stimulated cell extracts and serum-stimulated cell extracts, followed by that found in extracts from cells maintained in 15% serum. As shown in Fig. 7B, the in vitro stimulation seen in serum- and TPA-stimulated cell extracts is also effective on a template that contains only rDNA sequences between −150 and +32 (lacking the putative SRE).

In summary, the stimulation of *Drosophila* cells by serum or TPA treatment results in increased rRNA transcriptional activity present in nuclear extracts from these cells. This enhanced Pol I-specific activity requires sequences between −150 and −34 for the effect to be observed. The sequences between −60 and −34 support a moderate increase in transcriptional activity, while sequences between −60 and −150 augment transcriptional stimulation. These data suggest that an SRE-homologous sequence element present at approximately +558 does not contribute to the serum simulation that we observe. Our data also indicate that sequences from −150 to −34 are required for both serum and TPA stimulation of rDNA transcription.

DISCUSSION

In this study, we have demonstrated that serum and the tumor promoter TPA can stimulate the synthesis of rRNA at the transcriptional level. Nuclear extracts prepared from cells grown in serum-depleted medium (2% serum) show a very low level of transcriptional activity on an rDNA template containing sequences from −150 to +680 (Fig. 3).

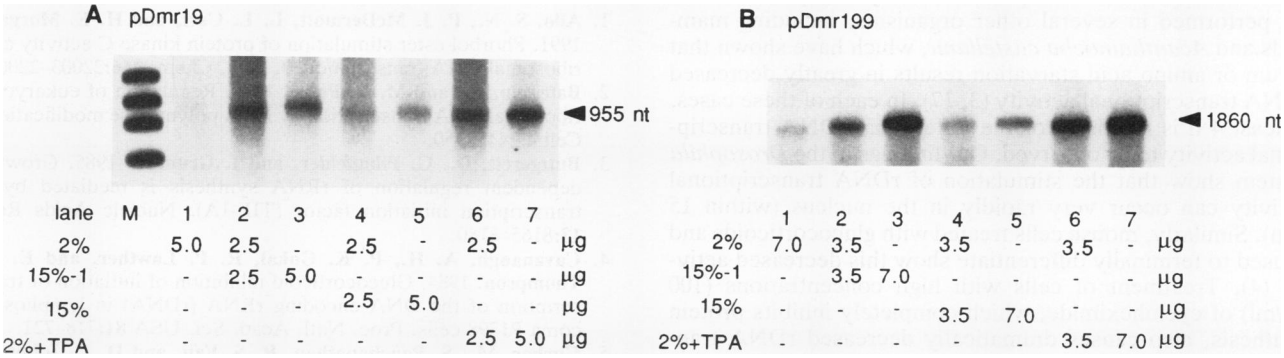

FIG. 7. Evidence that the stimulation of RNA polymerase I transcription results from the increased activity in cells treated with serum and TPA. Cell growth conditions and treatment were the same as for Fig. 4. The DNA templates were pDmr19 digested with *Sal*I (A) and pDmr199 digested with *Sca*I (B); 0.4 μg of DNA template was added in each transcription reaction. The total amount of mixed extract protein from untreated and treated cells is indicated below each lane. The untreated cells were cells grown in medium supplemented with 2 or 15% serum; the treated cells were cells grown in 2% serum and stimulated with serum for 1 h (15%-1) or treated with TPA (2%+TPA). The rRNA transcripts were analyzed as for Fig. 2. In panel A, lane 1 was exposed five times longer than were the other lanes.

Nuclear extracts prepared from cells grown in medium containing 2% serum and shifted to a medium containing 15% serum for 1 h contain a ninefold-higher level of activity. Similarly, cells maintained in medium containing 15% serum yield nuclear extracts with high transcriptional activity. These activities are insensitive to high levels (300 μg/ml) of α-amanitin and are therefore due to Pol I.

Cells grown in serum-depleted medium can also be stimulated by exposing them to TPA for 15 min. Nuclear extracts made from these cells show a 10-fold increase in Pol I transcriptional activity (Fig. 5). This very rapid increase in activity is reflected in a similar increase observed in nuclear run-on transcripts (27).

The Pol I transcriptional activity of extracts from serum-depleted cells can be increased by the addition of extracts from serum- or TPA-treated cells. The final level of activity in these mixed extracts is as great as that found in the treated cell extracts (Fig. 7). This result suggests that the higher transcription seen in treated cell extracts is caused by greater RNA Pol I activity and that the lower transcription observed in extracts from serum-depleted cells is not the result of a negative regulatory factor or inhibitor.

In comparing the activity of stimulated and serum-depleted cell extracts (Fig. 5 and Table 1) on different rDNA templates, we observed that the 5′ region (−43 to +32) is necessary for detection of a three- to fourfold-higher level of transcriptional activity in the treated cell extracts. However, a region farther upstream (between −60 and −150) further increases the stimulatory effect of treated cell extracts on rDNA transcription. The data in Fig. 5 and Table 1 also suggest that the region between −150 and −60 contains a regulatory element for both serum and TPA stimulation, although the core promoter region extending from −43 to +32 does support an intermediate level of stimulated transcription (three- to fourfold). It should be noted that basal-level transcription is not affected by 5′ deletions so long as sequences from −43 to +32 are present in the rDNA template. The initiation site for rDNA transcription was determined by S1 nuclease mapping (Fig. 4 and 6). The results show that the promoter upstream region to −34 can still support correct initiation. The upstream region to −150 is required for ninefold stimulation (Fig. 4). The template that contains only 5′ sequences to −34 yields a 20-fold decrease in basal-level transcription. In addition, this template cannot support the increased transcription seen with templates that contain sequences from −43 to −150.

Our results in the *Drosophila* rDNA system parallel studies performed in several other organisms, including mammals and *Acanthamoeba castellanii*, which have shown that serum or amino acid starvation results in greatly decreased rDNA transcriptional activity (3, 17). In each of these cases, at least 4 h is required for the differential rDNA transcriptional activity to be observed. Our findings in the *Drosophila* system show that the stimulation of rDNA transcriptional activity can occur very rapidly in the nucleus (within 15 min). Similarly, mouse cells treated with glucocorticoids and caused to terminally differentiate show this decreased activity (4). Treatment of cells with high concentrations (100 μg/ml) of cycloheximide, which completely inhibits protein synthesis, also causes dramatically decreased rDNA transcriptional activity (6, 26). The effect of cycloheximide on rDNA transcription can occur rapidly, within 30 min. The down-regulation of rDNA transcription by hormone treatment, cycloheximide treatment, or serum starvation parallels a change in the activity of an essential transcription factor found closely associated with Pol I or with Pol I itself (2, 13, 21, 25, 26). In total, these results indicate that regulation of rDNA transcription can occur rapidly as well as slowly and that this regulation can depend on external signals received by a given cell type.

Results presented here also demonstrate that the core promoter sequences from −43 to +4, which Kohorn and Rae (11) identified as necessary for basal-level transcription, can also support increased transcription in extracts from serum- or TPA-treated cells. However, maximal stimulation of Pol I activity is not observed until sequences to −150 are included in the template. As a result, *D. melanogaster* appears to fit the general model for rDNA promoters in that it contains both a core promoter sequence that can sustain basal levels of transcription and an upstream control element (within 200 bp of the start site of transcription), the presence of which results in higher levels of transcription (reviewed in references 8, 18, 23, and 24). It is interesting to note that a sequence found at approximately +558 shows 65% homology to the consensus sequence for mammalian SREs. However, we find that this sequence is not required to confer stimulation on an rDNA promoter in vitro (Fig. 5B and C). It was also demonstrated that in cultured rat cardiomyocytes, TPA stimulates rRNA synthesis and increases membrane-associated protein kinase C activity (1). It was recently shown that in the *Drosophila* system, RNA Pol III-transcribed genes demonstrate increased transcription in extracts made from TPA-stimulated cells (5). Therefore, the effect can be executed in different species and on different RNA polymerase activities. In summary, our findings demonstrate that *Drosophila* rDNA contains promoter elements, core and upstream, similar to those found in other organisms. These sequence elements support increased levels of transcriptional activity found in nuclear extracts from serum- and TPA-treated cells. Increased Pol I activity occurs within minutes following treatment, suggesting that rDNA transcription is rapidly regulated in response to extracellular signals. Our current work focuses on the isolation and characterization of the Pol I system components that mediate this transcription stimulation.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants GM-30443 and GM-40857.

We thank Bruce Kohorn for the gift of plasmids and Linda Reppas for preparation of the manuscript.

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