

# Metazoan rDNA Enhancer Acts by Making More Genes Transcriptionally Active

Yvonne N. Osheim,\* Edward B. Mougey,‡ Jolene Windle,‡ Mary Anderson,‡ Marina O'Reilly,§ Oscar L. Miller, Jr.,§ Ann Beyer,\* and Barbara Sollner-Webb‡

\*Department of Microbiology, University of Virginia School of Medicine, Charlottesville, Virginia 22908; ‡Department of Biological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205; and §Department of Biology, University of Virginia, Charlottesville, Virginia 22908

**Abstract.** Enhancers could, in principle, function by increasing the rate of reinitiation on individual adjacent active promoters or by increasing the probability that an adjacent promoter is activated for transcription. We have addressed this issue for the repetitive metazoan rDNA enhancer by microinjecting *Xenopus* oocytes with enhancer-less and enhancer-bearing genes and determining by EM the frequency that each gene type forms active transcription units and their transcript density. We use conditions where transcription requires the normal rDNA promoter and is stimulated 30–50-fold by the enhancer. (In contrast, at saturating template conditions as used in previous EM studies, an aberrant mode of transcription is activated that is not affected by the rDNA enhancer or by the generally rec-

ognized rDNA promoter.) The active transcription units on enhancer-less genes are found to be as densely packed with nascent transcripts and polymerases as those on enhancer-bearing genes and on the endogenous rRNA genes. Significantly, the enhancer-bearing genes are ~30–50-fold more likely to form such active transcription units than enhancer-less genes, consistent with their amounts of transcript. Complementary studies confirm that the enhancer does not affect elongation rate, the stability of the transcription complex, or transcript half-life. These data demonstrate that the repetitive metazoan rDNA enhancer causes more genes to be actively transcribed and does not alter the reinitiation rate on individual active genes.

**E**NHANCERS are DNA elements that markedly increase the total amount of transcript from promoters located in *cis*, functioning over distances and in both orientations. Despite an increasing knowledge of the proteins that bind to enhancers of genes transcribed by RNA polymerase II (pol II)<sup>1</sup> and their frequent interactions with various of the basal pol II transcription factors, it remains unclear how enhancers actually augment transcription and whether different enhancers may operate by fundamentally different mechanisms. A central unresolved question about enhancers is whether they function by increasing the expression level from individual active

*cis*-located promoters (Fig. 1 A) or by increasing the chance that a *cis*-located promoter will be activated (Fig. 1 B). In the former scenario the enhancer would increase the reinitiation frequency on individual active genes but not affect the total number of active genes, while in the latter scenario the enhancer would increase the total number of those promoters in a culture that are transcribing (increasing the number that become activated or the duration they remain active) and not alter the expression level from each transcribing gene.

For enhancers of genes transcribed by pol II, both models are popularly considered. Data showing that pol II enhancers favor binding of TFIID to the adjacent promoter (e.g., Abmayr et al., 1988; Horikoshi et al., 1988; Workman et al., 1988; for reviews see Lewin, 1990; Ptashne and Gann, 1990; Klein and Struhl, 1994) and increase the fraction of cells containing the protein product from a transfected gene if its promoter carries an enhancer in *cis* (Weintraub, 1988; White et al., 1992; Walters et al., 1995) indicate that these enhancers function by increasing the chance that the adjacent promoter will be transcribed (as in Fig. 1 B). However, evidence that enhancers interact with pol II, TFIIF, and TFIIB which evidently recycle at

Yvonne N. Osheim and Edward B. Mougey contributed equally to this work.

Address all correspondence to Dr. Barbara Sollner-Webb, Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, MD 21205. Tel.: (410) 955-7419. Fax: (410) 955-0192. J. Windle's current address is Cancer Therapy and Research Center, San Antonio, TX 78229. M. O'Reilly's current address is Department of Biology, Johns Hopkins University, Baltimore, MD 21218.

1. *Abbreviations used in this paper:* pol I and pol II, RNA polymerases I and II, respectively.

each round of transcription (Roberts et al., 1993; Zawel and Reinberg, 1993; Xiao et al., 1994; Gerber et al., 1995; Zawel et al., 1995; and references therein), as well as many investigators' feelings that enhancers of single copy genes should activate similarly in each cell of a tissue, instead fuel consideration of a model where pol II enhancers increase the transcription reinitiation frequency on each adjacent active promoter (as in Fig. 1 A; see Kingston and Green, 1994).

Enhancers also exist for rRNA genes transcribed by RNA polymerase I (pol I) (for reviews see Reeder, 1984, 1992; Sollner-Webb and Tower, 1986; Paule, 1994; Jacob, 1995; Moss and Stefanovsky, 1995). In numerous metazoan organisms (including frog, mouse, hamster, rat, fly, and *Arabidopsis*), and evidently also in certain unicellular organisms (including *Acanthamoeba*), rDNA enhancers are repetitive elements that extend over the ~2 kb upstream from the gene promoter. These enhancers stimulate promoters in *cis* and inhibit unlinked promoters in *trans*, suggesting that they bind a transcription factor in common with the promoter (Labhart and Reeder, 1984; Pape et al., 1989). A specific footprinting pattern of the pol I transcription factor UBF on rDNA enhancers (Pikaard et al., 1989, 1990) suggested that it mediates enhancer function, but the low sequence-selectivity of UBF binding (Pikaard et al., 1989; Copenhagen et al., 1994; Hu et al., 1994) and mutational analysis (Pikaard, 1994) raises questions about whether it is the only or even a critical player in enhancement.

The action of rDNA enhancers was first identified and has been most extensively documented using *Xenopus* oocyte microinjection, biochemically analyzing the amount of resultant transcript (Moss, 1983; Reeder et al., 1983; Labhart and Reeder, 1984, 1985; Dunaway and Droge, 1987; Pikaard and Reeder, 1988; Pape et al., 1989; Pikaard et al., 1990). Notably, in all these enhancer studies, the rDNA was microinjected at  $\leq 0.3$  fmole promoter per oocyte nucleus, usually at  $< 0.03$  fmole per oocyte nucleus. Enhancer-bearing and enhancer-less promoter constructs generally were coinjected into the same oocyte in competition, either at equimolar amount (e.g., Moss, 1983; Reeder et al., 1983; Labhart and Reeder, 1984, 1985; Pikaard and Reeder, 1988; Pape et al., 1989; as in Fig. 2 E and F) or using a substantial excess of the enhancer-less gene to partially equalize the total amount of transcript from the two genes (e.g., Reeder et al., 1983; Labhart and Reeder, 1984; as in Figs. 4–6). A stimulatory effect of *cis*-located enhancers is also apparent in single microinjections (Pape et al., 1989; as in Fig. 2 A and B). Furthermore, results on the *Xenopus* enhancer are likely applicable to other metazoan species, since *Xenopus* rDNA enhancers can function interchangeably with mouse and *Arabidopsis* rDNA enhancers, both in intact cells and in cell extracts (Kuhn et al., 1990; Pikaard et al., 1990; Doelling et al., 1993).

Published data do not resolve whether the repetitive rDNA enhancer functions by increasing the transcriptional level from each activated rRNA gene (Fig. 1 A) or by causing a larger fraction of the genes to be transcribed (Fig. 1 B) (for review see Moss and Stefanovsky, 1995). Even the biochemical finding from competition experiments that enhancers must act early in the transcription reaction (Labhart and Reeder, 1984; Dunaway and Droge,

1987; Pape et al., 1989) is consistent both with enhancers increasing the number of activated genes (Labhart and Reeder, 1985; Pikaard and Reeder, 1988) and with enhancers causing the adjacent promoter to bind polymerase and initiate more efficiently, not altering the number of active genes (Moss, 1983; DeWinter and Moss, 1987; Mitchelson and Moss, 1987).

Electron microscopic analysis of ribosomal transcription units appears well suited to directly distinguish whether the repetitive rDNA enhancer functions by making individual genes more active or by activating more genes. In most, but significantly not all, visualizations of cellular or microinjected rRNA genes bearing their natural enhancers, the nascent transcripts and associated pol I molecules are packed very densely, every ~100 bp along the active genes (Miller and Beatty, 1969; Trendelenburg and Gurdon, 1978; but see Foe, 1978; for reviews see Miller, 1981 and Mougey et al., 1993). We early on observed a similar high polymerase density on active deleted genes (Bakken et al., 1982a,b) that turned out to contain the complete *Xenopus* rDNA promoter and only one enhancer repeat following their microinjection into *Xenopus* oocytes. This observation was taken to indicate that the normal rDNA promoter alone directs dense polymerase packing and consequently that the enhancer must cause formation of more transcription complexes (Busby and Reeder, 1983; Reeder et al., 1993; Reeder, 1984; Labhart and Reeder, 1984, 1985; Pikaard et al., 1989). It was, however, somewhat troubling that no transcriptional stimulation by the enhancer region was noted in those experiments (Bakken et al., 1982a,b). That could imply that the experimental conditions were not suitable to study enhancer action or that the enhancer might act by affecting processes that were not detectable by the microscopy, such as elongation rate or transcript half-life.

Indeed, reconsideration of published data, in conjunction with the new results of Fig. 2 below, indicates that the early electron microscopic observations (Bakken et al., 1982a,b) do not address either the action of the normal rDNA promoter or the process of transcriptional enhancement. Those microinjections were performed at very high template concentration (injecting 3–6 fmoles promoter per oocyte nucleus, >10 times more than has been used to study the enhancer's effect biochemically), and Fig. 2 below will demonstrate that this transcription is not affected by enhancer sequences. In fact, injection of such high amounts of rDNA template activate an aberrant mode of transcription that is directed by only an ~10-bp initiator element (Sollner-Webb et al., 1983) and is not affected by template concentration (Sollner-Webb and McKnight, 1981). Because that transcription is not responsive to the entire upstream portion of the otherwise essential 140-bp rDNA promoter (Windle and Sollner-Webb, 1986a; see also Fig. 2 A and B, lanes 2–4) which normally binds the essential pol I transcription factors SL1 and UBF (Learned et al., 1986; Tower et al., 1986; Bell et al., 1988; Pikaard et al., 1989), its initiation must occur by rather different means than the known transcription factor interactions. The previous EM observations (Bakken et al., 1982a,b) presumably address only this aberrant mode of initiation that is independent of the normal promoter and is unresponsive to the enhancer, so they do not answer whether the repeti-

tive rDNA enhancer acts by making active genes more active or by increasing the number of active genes (Fig. 1).

We have now studied the effect of the rDNA enhancer using oocytes injected with subsaturating amounts of rDNA template, where transcription requires the complete promoter and is stimulated by the enhancer. Electron microscopic visualizations of transcribing chromatin assembled on thus injected enhancer-bearing and enhancer-less genes demonstrate that the *Xenopus* rDNA enhancer markedly increases the number of active genes that are observed. It does not affect the density of nascent transcripts on the active rRNA genes, the transcriptional elongation rate, or the half-life of the resultant transcript. Therefore, under conditions where the normal rDNA promoter is used and the enhancer stimulates transcription, the enhancer functions by increasing the fraction of *cis*-located promoters that are actively transcribing.

## Materials and Methods

### Plasmid Constructs

The *X. laevis* rDNA plasmids 5' $\Delta$ -1150, 5' $\Delta$ -255, 5' $\Delta$ -127, and 5' $\Delta$ -65, containing the initiation region from the designated position to +115, were described (Sollner-Webb et al., 1983; Windle and Sollner-Webb, 1986a). The E plasmid, containing 10 copies of the 60/81-bp enhancer repeat, and the A plasmid, containing the *X. laevis* rDNA region -245 to +13 joined to a 79-nt tag sequence, were described in Pape et al. (1989); the B plasmid is like the A plasmid but using a different 42-nt tag sequence, as described (Windle and Sollner-Webb, 1986b). The EA6 gene is like the EA293-3' gene of Mougey et al. (1993) except the rRNA segment beyond the tag sequence extends to +729. Specifically, it contains from -1150 to +13 (including the E enhancer block) joined to the A tag sequences, followed by rDNA residues +28 to +729 (to direct terminal ball formation on the transcript) and then the last 2/3 (5.1 kb) of the rDNA coding region and a transcriptional terminator (see Fig. 3). The A6 gene is analogous to the EA6 gene but lacks the 60/81-bp enhancer repeats. The EB12 gene and the B12 gene are like the EA6 and A6 gene except they contain the B instead of the A tag sequence just downstream from the initiation site and contain two tandem copies of the 5.8-kb rRNA coding region present in the A6 gene (see Fig. 3).

The enhancer-less and enhancer-bearing mouse rDNA genes used for the experiment of Fig. 7 were 5' $\Delta$ -230 and 5' $\Delta$ -1800 of Pikaard et al. (1990), containing the indicated rDNA position to +292 and linearized with NdeI 514 nt beyond the initiation site. The long run-off enhancer-bearing and enhancer-less genes used for the experiment of Fig. 8 were -2150 and -2150: $\Delta$ E:pBR of Paalman et al. (1995), containing the complete -2150 to +292 sequence or that region with the -1800 to -231 enhancer block replaced by 1.5 kb of pBR322; they were linearized with PstI 2 kb beyond the initiation site.

### Oocyte Injections

Oocytes were isolated manually from excised sections of *Xenopus* ovary and ~40 nl of plasmid DNA in 10 mM Tris-HCl (pH 7.6), 0.1 mM EDTA, 200  $\mu$ g/ml  $\alpha$ -amanitin was injected into the germinal vesicle of each, as described (Mougey et al., 1993). The amounts of injected DNA are given in the text. After 24-h incubation at 18°C (see Figs. 4-6) or 8-24 h (Fig. 2), RNA was prepared from ~20 pooled oocytes for each injected plasmid, and one oocyte's worth was subjected to S1 nuclease analysis as described (Mougey et al., 1993; Sollner-Webb and McKnight, 1982). All injections were performed in duplicate (only one sample of which is shown) and all experiments were repeated more than one time, yielding very reproducible results. The isolated single strand S1 probes for the A and B genes were 5' end labeled in the tag region, 97 or 60 nts, respectively, downstream from the initiation site (Pape et al., 1989); S1 analysis of the 5' $\Delta$  series used the 5' end labeled single stranded H probe, a HinI fragment labeled at +55 (Sollner-Webb et al., 1983). For the experiments of Figs. 4-6, additional oocytes from each set of injections were subject to chromatin dispersal at 24 h postinjection, spreading 1 oocyte nucleus per grid, and were visualized by EM, as described (Mougey et al., 1993). In these

spreads, the transcribing plasmids can be readily distinguished due to the size of their transcription units (6 kb for the A and EA genes vs. 12 kb for the B and EB genes) and can be confirmed by their overall plasmid lengths (which differ by 6 kb for the coinjected A and EB pair and by 4 kb for the EA and B pair).

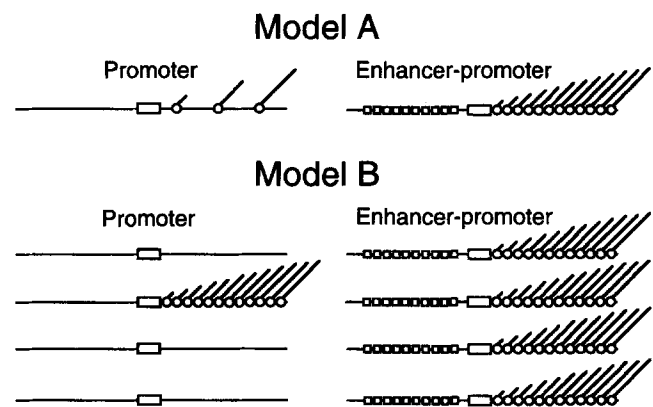
## In Vitro Transcription Analysis

Preparation of S-100 extract from mouse L1210 cells and in vitro transcription analyses were as described in Miller and Sollner-Webb (1981) and modified in Paalman (1995) and Paalman, M.H., J. Wilkinson, and B. Sollner-Webb (manuscript in preparation). The 25- $\mu$ l reactions used ammonium sulfate precipitated extract, 2.5 fmol of template plasmid supplemented with 25 ng pBR322 plasmid, and the incubation times described in the text.

## Results

### Determination of Transcription Conditions Suitable for Electron Microscopic Analysis of Enhancer Action

We first wanted to define experimental conditions in which to best analyze the action of the repetitive rDNA enhancer by oocyte microinjection and subsequent electron microscopic visualization of transcribing enhancer-bearing and enhancer-less genes. The desire is to use as high an amount of injected rDNA as possible, to maximize detection of transcriptionally active molecules, yet to work under conditions where the enhancer has its large stimulatory effect on the level of normal transcription. Electron microscopic analyses of transcription complexes on rRNA gene constructs have routinely used oocytes injected with >3 fmol promoter per nucleus (e.g., Bakken et al., 1982a,b; Morgan et al., 1982). However, injection of  $\geq$ 4 fmol promoter per nucleus was subsequently found to activate the aberrant mode of rDNA transcription which is directed by a very small DNA element and is unresponsive to the recognized transcription factor binding domains of the 140-bp rDNA promoter that are absolutely needed when injections are performed at  $\leq$ 2 fmol promoter per oocyte nucleus (Sollner-Webb et al., 1983; Windle and Sollner-Webb, 1986a,b; Fig. 2, A and B, lanes 2-4). Because action of the rDNA enhancer had only been demonstrated using



**Figure 1.** Models of rDNA enhancer action. An enhancer could increase the polymerase reinitiation frequency on individual active genes (*Model A*) or could increase the chance that an adjacent promoter is transcribed (*Model B*). □, enhancer; ◻, promoter; ○, transcribing polymerases; and //, nascent transcripts.

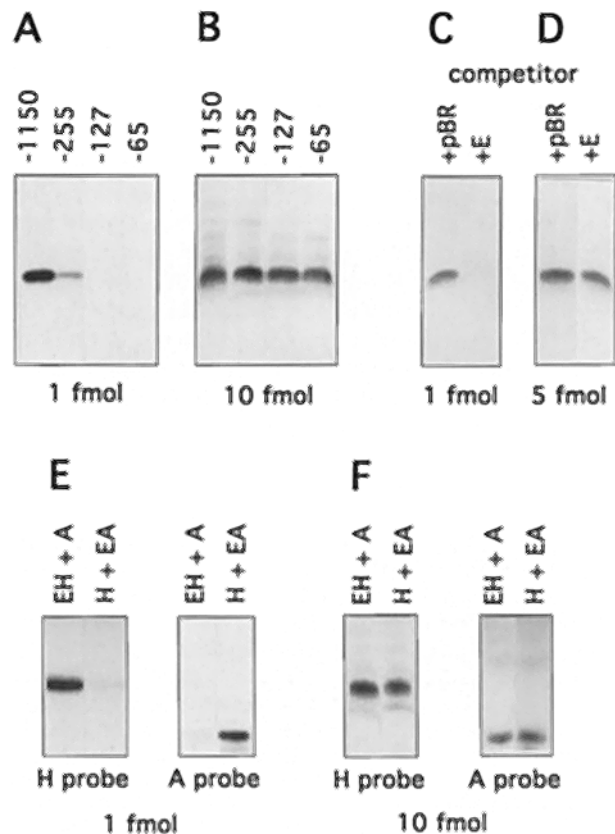
low injected template concentration (e.g., Reeder et al., 1983; Labhart and Reeder, 1984, 1985; Pape et al., 1989), we assessed the enhancer at the high injected promoter concentration. None of the normal effects of the enhancer is observed on genes microinjected at  $\geq 4$  fmol promoter per oocyte nucleus (Fig. 2, and data not shown). In single injections, the rDNA enhancer does not stimulate transcription of a promoter *in cis* (Fig. 2 B; lanes 1–2, and data not shown), although it stimulates greatly when injected at  $\leq 2$  fmol promoter per oocyte (Fig. 2 A, lanes 1–2, and data not shown). Similarly, a subcloned enhancer inhibits transcription from an equimolar amount of a coinjected rDNA promoter (e.g., Labhart and Reeder, 1984; Pape et al., 1989) at  $\leq 2$  fmol injected plasmid per oocyte (Fig. 2 C, and data not shown), but inhibition does not occur when the plasmids are coinjected at  $\geq 4$  fmol per oocyte (Fig. 2 D, and data not shown). This failure of rDNA initiation to be competed by enhancer repeats *in trans* adds support to the hypothesis that initiation at these saturating template concentrations does not use the same limiting factor(s) that is presumably sequestered by the enhancer *in trans* (Labhart and Reeder, 1984) and is important for initiation when the usual promoter recognitions are operative.

A similar template concentration dependence is also observed in the rDNA enhancer assay that involves coinjecting oocyte nuclei with equimolar amounts of two marked plasmids, one containing an enhancer-less gene and the other containing an enhancer-bearing gene (Fig. 2, E and F). When introduced at total promoter concentrations of  $\leq 2$  fmol per oocyte, the enhancer-bearing promoter (designated with an E) out competes the enhancer-less promoter (designated as H or A) by over an order of magnitude (Labhart and Reeder, 1984; Fig. 2 E, and data not shown). In contrast, when these pairs of constructs are coinjected at  $\geq 4$  fmol promoter per nucleus, the enhancer-bearing and enhancer-less templates yield similar amounts of transcript (Fig. 2 F, and data not shown). Thus all the known affects of the rDNA enhancer that are operative at low injected promoter concentration are not observed at high promoter concentration.

When oocytes were coinjected with low amounts of rDNA plasmids plus excess pBR322 plasmid to bring the total DNA concentration to the high promoter concentration conditions, the transcription still required the normal large promoter and was stimulated by the enhancer *in cis* (Windle and Sollner-Webb, 1986a, and data not shown). This indicates that promoter concentration and not total injected DNA concentration is the critical factor in determining whether transcription occurs independent of the normal promoter and enhancer.

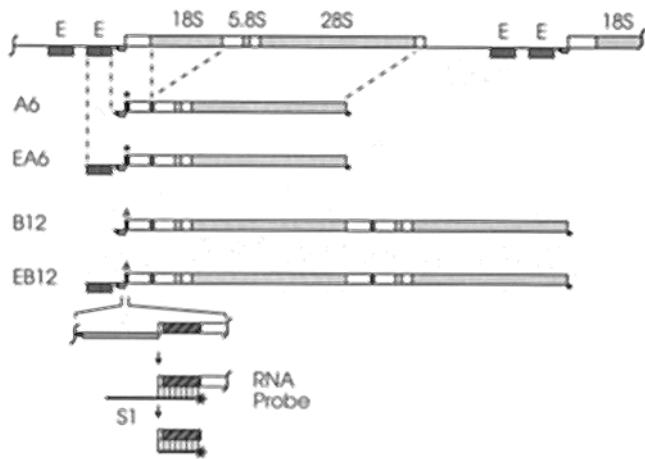
### Electron Microscopic Analysis of Enhancer Action

We next used electron microscopic visualization of transcribing enhancer-bearing and enhancer-less rDNA genes injected into *Xenopus* oocytes under enhancer-responsive conditions to assess whether the rDNA enhancer functions by causing each transcribing gene to be more active (presumably increasing its polymerase density from a low to a high level) or by increasing the total number of transcribing genes (Fig. 1). We prepared the large enhancer-less genes A6 and B12 and similarly prepared their enhancer-



**Figure 2.** Enhancer effects at high and low concentration of microinjected promoter. (A and B) *Cis* assay. An enhancer-bearing *X. laevis* rRNA minigene (5'Δ-1150) or enhancer-less derivatives that contain the complete promoter (5'Δ-255) or lack the upstream promoter domain (5'Δ-127 and 5'Δ-65) was singly microinjected into *X. borealis* oocyte nuclei at 1 fmol per oocyte (A) or at 10 fmoles per oocyte (B). In this and the subsequent panels, the resultant transcripts were quantitated by S1 nuclease analysis. (C and D) *Trans* assay. Plasmid bearing the 5'Δ-255 minigene and either an equimolar amount of plasmid bearing a subcloned block of enhancer repeats (E) or the same amount of the pBR322 vector DNA were coinjected into *X. borealis* oocyte nuclei at 1 fmol per oocyte (C) or at 5 fmol per oocyte (D). (E and F) Competition assay. The indicated pairs of cloned enhancer-bearing and enhancer-less *X. laevis* rRNA minigenes were coinjected into *X. borealis* oocytes at 1 fmol total promoter per oocyte (E) or at 10 fmol total promoter per oocyte (F). EH and H are the 5'Δ-1150 and 5' Δ-255 plasmids whose transcripts are detected using the H probe; EA and A contain the same rDNA enhancer and promoter regions but transcribe a different sequence that is detected using the A probe. Analogous results to those of A–F were obtained upon injection into *X. laevis* oocytes (see also Fig. 4). Results like those in A, C, and E are obtained at approximately  $\leq 2$  fmol promoter per oocyte; results like in B, D, and F are obtained at approximately  $\geq 4$  fmol promoter per oocyte.

bearing derivatives EA6 and EB12 in which the enhancer block was inserted at its natural position (diagrammed in Fig. 3, top). The transcripts of these constructs differ in length (6 and 12 kb, respectively) so they can be readily distinguished by electron microscopy; they also contain the A or B 5' tag sequence so they can be quantitated by S1 nuclease analysis (Fig. 3, bottom; Pape et al., 1989). These marked enhancer-bearing and enhancer-less genes were coinjected into *Xenopus* oocytes at  $< 2$  fmol total pro-

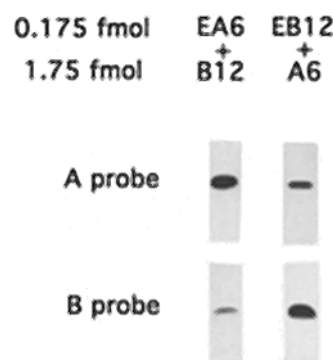


**Figure 3.** Plasmid constructs for EM. Genomic *X. laevis* rDNA is diagrammed above, with the coding region boxed and the enhancer repeats (*E*) indicated by vertical lines. The A6 and EA6 genes contain the complete promoter or the enhancer and promoter, respectively, and at residue 13 have inserted a 79-nt tag A sequence (\*; see Materials and Methods). Transcription then reads into the rest of the 5' ETS (nt 28–729), followed by a 5.15-kb segment that comprises the final 2/3 of the 40S pre-rRNA coding region and a pol I transcription terminator, generating a 6-kb (6,067 nt) transcription unit. The B12 and EB12 genes are analogous except they carry a different tag B sequence of 42 nt ( $\Delta$ ), and the 5.8-kb rRNA coding segments are tandemly duplicated, generating a 12 kb (11,960 nt) transcription unit. Below is an enlargement of the initiation region and a diagram of the S1 nuclease analysis of the transcripts. The transcribed region is boxed, the tag segment shown by diagonal hatches, and the S1 probe shown by a heavy line, its 5' end label indicated by the star.

motor concentration, and at 24 h postinjection half the oocytes of each dish were spread for electron microscopy while the other half were analyzed by S1 nuclease mapping to biochemically verify the effect of the enhancer.

In preliminary experiments, we coinjected equimolar low amounts of enhancer-less and enhancer-bearing plasmids. The S1 analyses showed  $\sim 30$ – $50$ -fold more transcripts from the enhancer-bearing gene than from the enhancer-less gene, and the electron microscopic analysis showed only fully packed transcription units on the active genes, virtually all on the enhancer-bearing construct (data not shown). To allow more convenient electron microscopic visualization of appreciable numbers of transcribing enhancer-less genes, we turned to the modified injection protocol where 10-fold more of the enhancer-less gene than the enhancer-bearing gene is injected (introducing 1.75 and 0.175 fmol per oocyte, respectively). Analysis using such unequal amounts of injected enhancer-less and enhancer-bearing genes (Reeder et al., 1983; Labhart and Reeder, 1984) is possible because the amount of transcript is proportional to the amount of injected plasmid and the magnitude of the enhancer effect remains constant at  $\leq 2$  fmol plasmid per oocyte (Sollner-Webb and McKnight, 1981, and data not shown).

When oocytes were so coinjected with the EA6 and B12 genes, the S1 nuclease analysis using a constant amount of oocyte RNA showed 3–5-fold more EA6 transcript than B12 transcript (Fig. 4, left lanes, and data not shown). Since

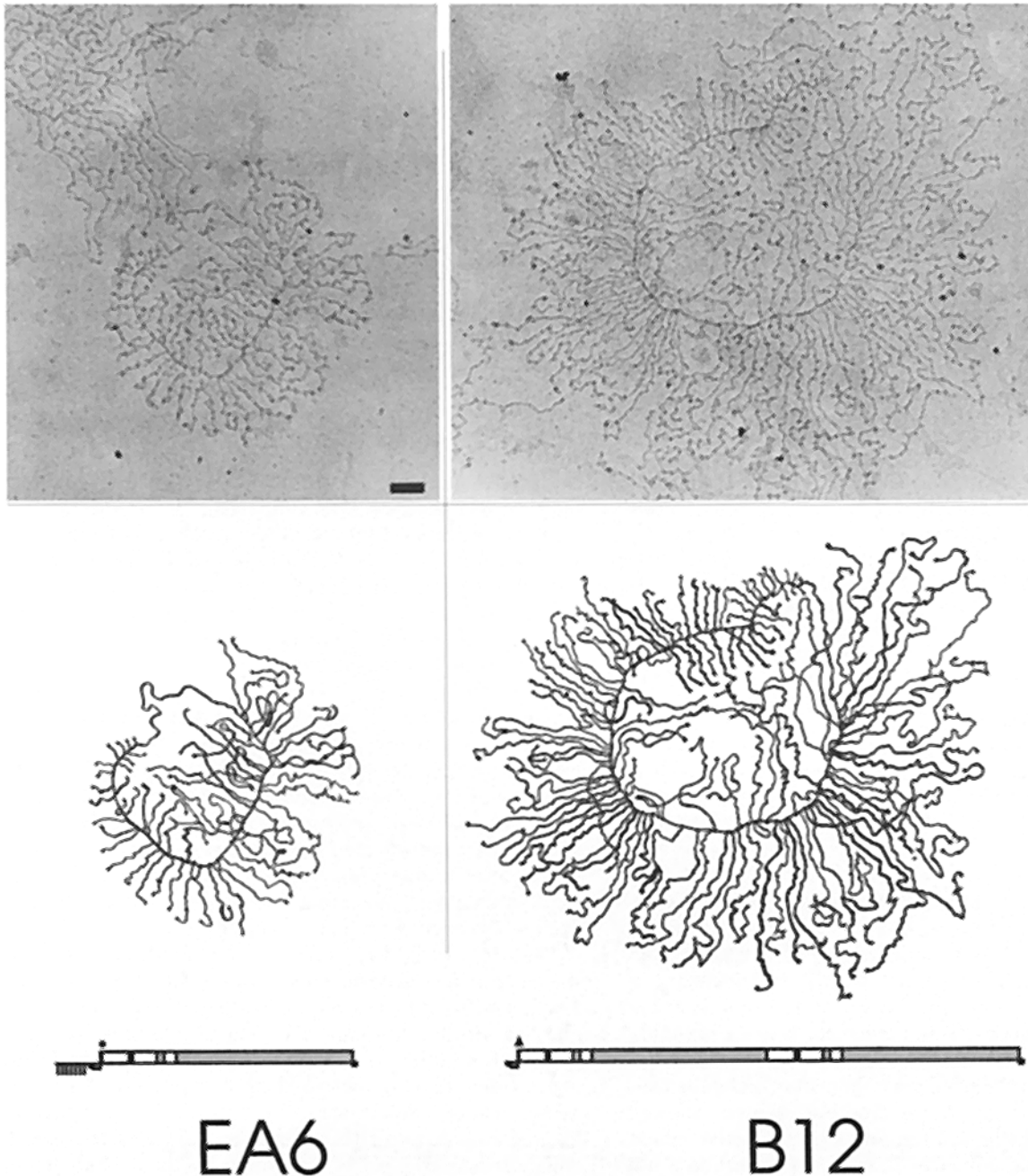


**Figure 4.** S1 nuclease analysis of RNA from oocyte coinjections. 0.175 fmol of EA6 plasmid plus 1.75 fmol of B12 plasmid (also used for Fig. 5) or 0.175 fmol of EB12 plasmid plus 1.75 fmol of A6 plasmid (also used for Fig. 6, *A* and *B*) were coinjected into *X. laevis* oocytes. 10-fold more enhancer-less than enhancer-bearing gene was injected, and the total promoter concentration was maintained at  $< 2$  fmol per oocyte. One oocyte's worth of each pooled RNA preparation was subjected to S1 nuclease analysis, using excess probes specific for the A or the B gene transcript.

10-fold less EA6 gene than B12 gene was microinjected, the EA6 gene transcribed  $\sim 30$ – $50$ -fold more efficiently than the B12 gene, on a per gene basis. To confirm that no bias was introduced by the different lengths of the transcription units or the differently tagged sequences of the A6 and B12 genes, the experiment was also performed in reverse, coinjecting the enhancer-less A6 gene and enhancer-bearing EB12 gene, again at a 10:1 molar ratio of the enhancer-less to the enhancer-bearing gene. The S1 nuclease analyses again showed 3–5-fold more EB12 than A6 transcript (Fig. 4, right lanes, and data not shown), demonstrating that the EB12 gene transcribed  $\sim 30$ – $50$ -fold more efficiently than the A6 gene, on a per gene basis. Thus the enhancer was functioning efficiently. When similar injections were performed using a fourfold lower amount of total injected gene, we observed no greater preferential transcription of the enhancer-bearing gene (data not shown), indicating that the injection conditions of Figs. 4–6 elicit the full enhancer effect.

The other half of the injected oocytes from each dish was spread for electron microscopy (Miller and Beatty, 1969). Transcribing molecules visualized from oocytes coinjected with the EA6 and B12 pair of plasmids are typified by those of Fig. 5. The active enhancer-bearing EA6 genes (identified by their 6-kb transcription units) were densely packed with nascent transcripts and polymerases, like the endogenous rRNA genes. Significantly, the active enhancer-less B12 genes (identified by their 12-kb transcription units) were similarly densely loaded with nascent transcripts and polymerase (Fig. 5). Complementary results were obtained from spreads of the oocytes coinjected with the A6 and EB12 pair of plasmids (Fig. 6 *A*). Again, the transcription units on both the active enhancer-bearing EB12 genes (identified by their 12-kb transcription units) and the active enhancer-less A6 genes (identified by their 6-kb transcription units) were densely packed with nascent transcripts.

The density of the nascent transcripts was quantitated by tracing and counting 10 representative micrographs of each of the active gene types from oocytes coinjected with the EA6 and B12 pair of genes and from ones coinjected with the A6 and EB12 pair of genes, as well as of endogenous rRNA genes. Dividing the average numbers of transcripts per gene by the sizes of the respective transcription units, the average number of base pairs per nascent tran-



**Figure 5.** Electron micrographic analysis of transcription units from coinjected oocytes. Oocytes coinjected with 0.175 fmol of EA6 plasmid and 1.75 fmol of B12 plasmid (see Fig. 4, left) were spread (Miller and Beatty, 1969) for EM. The EA6 and B12 genes were identified by the length of their transcription units, and confirmed by measuring the plasmid lengths. Representative micrographs are shown above, with tracings shown below. The template is diagrammed in Fig. 3. Bar, 0.2  $\mu\text{m}$ .

script and associated polymerase was calculated (Table I, right columns). Within the margin of error, these values were the same for the enhancer-bearing gene and the enhancer-less gene in each pair of coinjections and for the endogenous rRNA genes; all are  $\sim 100$  bp per transcribing polymerase (Table I). This value is consistent with previous measurements of polymerase packing on endogenous amphibian rRNA genes (e.g., Miller and Beatty, 1969). Thus, under enhancer-responsive conditions, a high density of nascent transcripts is a property of the active rDNA promoter

and does not require the rDNA enhancer. This indicates that the enhancer must function other than by modulating the density of transcribing polymerases on each active gene.

There was one major difference between the visualizations of the enhancer-bearing and the enhancer-less genes—the observed number of active transcription units. Although 10-fold less enhancer-bearing gene than enhancer-less gene was microinjected, we observed 2–5 times more of the active enhancer-bearing gene than the active enhancer-less gene in both pairs of coinjections. Results from three



A

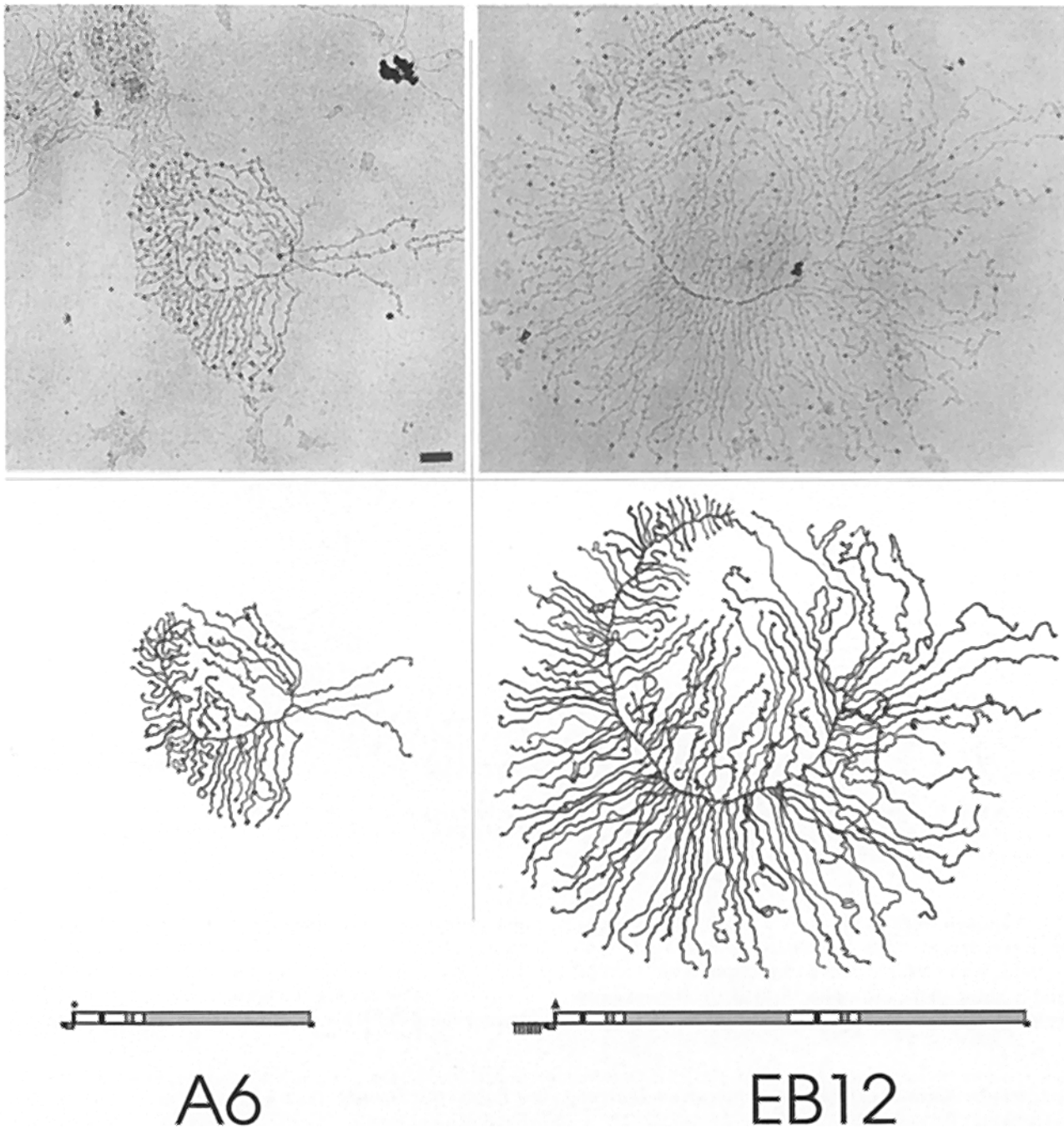
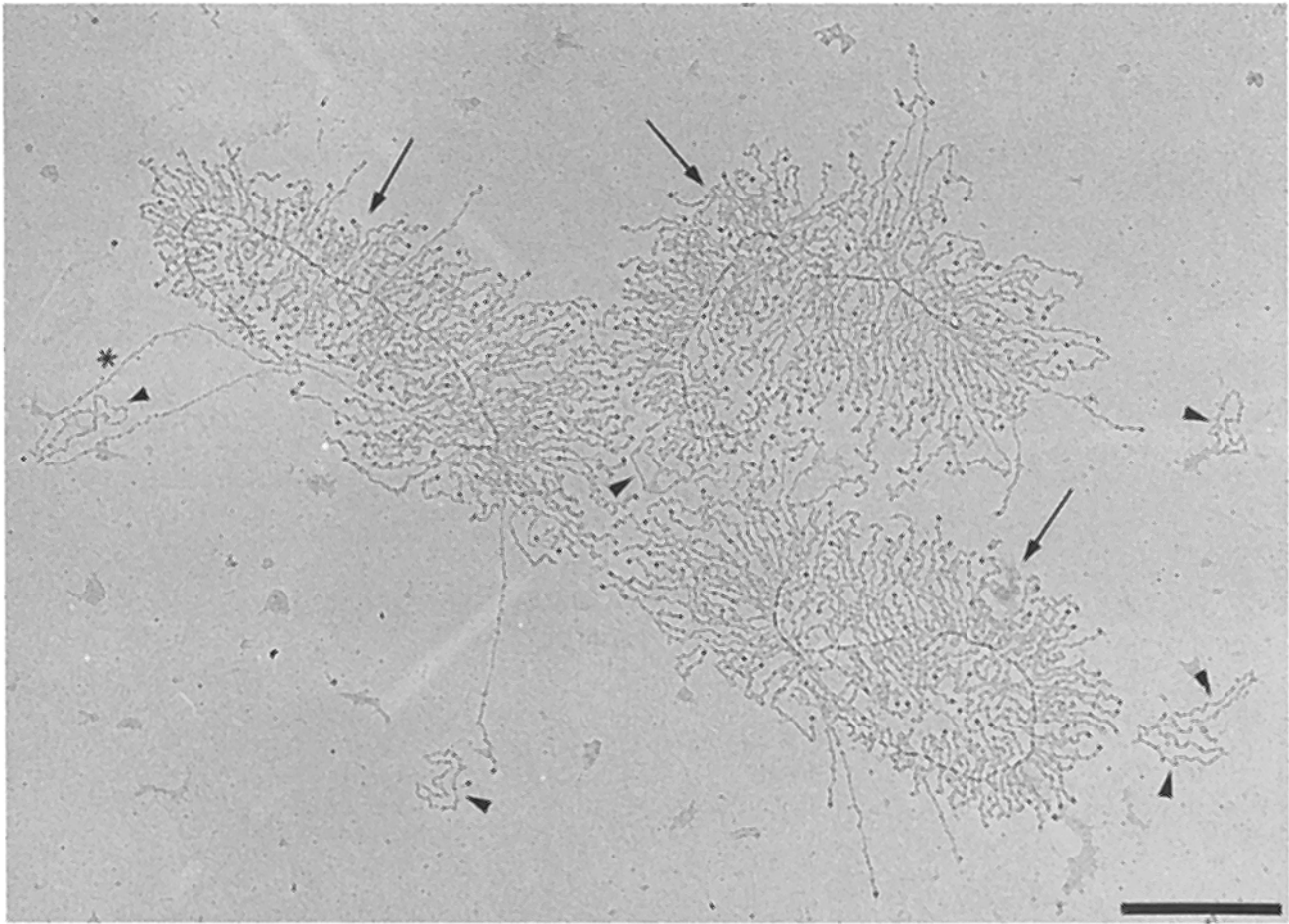


Figure 6 A. Electron micrographic analysis of transcription units from coinjected oocytes. Oocytes coinjected with 0.175 fmol of EB12 plasmid and 1.75 fmol of A6 plasmid (Fig. 4, right) were spread (Miller and Beatty, 1969) for EM and analyzed as in Fig. 5. A shows a representative of each active plasmid, with tracings below. The template is diagrammed in Fig. 3. Bar, 0.2  $\mu\text{m}$ .

of the injected frogs are shown in the middle columns of Table I. Thus, on a per gene basis, transcription units are observed  $\sim 20$ – $50$ -fold more frequently on enhancer-bearing plasmids than on coinjected enhancer-less plasmids. This critical result demonstrates that the rDNA enhancer stimulates expression by increasing the number of rRNA gene promoters that are transcriptionally active. It is also consistent with the above noted finding that virtually all

the active transcription units were on the enhancer-bearing gene when coinjected with the enhancer-less promoter at equimolar concentration.

The greater number of active enhancer-bearing genes than active enhancer-less genes observed in these experiments is illustrated by the electron micrograph of Fig. 6 B. This field includes three active EB12 genes (arrows) but only inactive copies of the coinjected A6 genes (arrow-

**B**

**Figure 6 B.** A field from the coinjection of Figure 6 A is shown. It contains three active EB12 plasmids (*arrows*) as well as inactive EB12 (*asterisk*) and inactive A6 (*arrowheads*) plasmids. Bar, 1  $\mu$ m. (Transcribing rDNA is known to be more extended than inactive nucleosome-covered DNA [Trendelenburg and Gurdon, 1978]).

*heads*). Although most fields show a much lower ratio of active transcription units to inactive plasmids of both kinds, fields with multiple active transcription units almost invariably show more examples of active enhancer-bearing gene than of active enhancer-less genes. If the en-

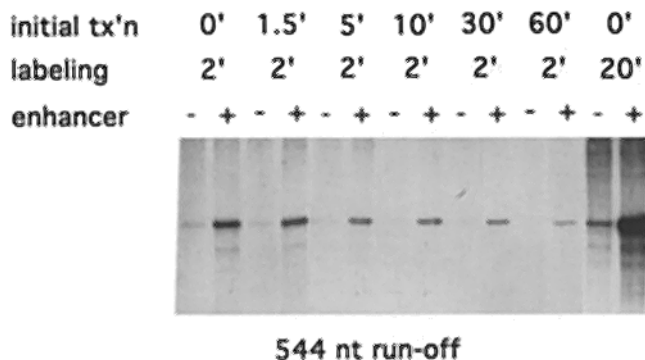
hancer-bearing and enhancer-less genes assembled active transcription units with equal efficiency, the majority of the active genes in virtually all such fields should have been the enhancer-less constructs, due to their 10-fold greater numbers. This underscores the greater propensity

**Table I. Relative Numbers of Observed Transcription Units and Their Polymerase Density**

Coinjected constructs	Ratio injected	Number TU observed in frog			Number TU counted	Pol/TU	bp of TU/pol
		a	b	c			
EA6	1	15	10	—	10	59 ( $\pm 4$ )	104 ( $\pm 6$ )
B12	10	3	5	—	10	108 ( $\pm 12$ )	110 ( $\pm 13$ )
A6	10	—	—	24	10	52 ( $\pm 5$ )	117 ( $\pm 11$ )
EB12	1	—	—	66	15	116 ( $\pm 31$ )	109 ( $\pm 29$ )
(endogenous)	—	>100	>100	>100	15	76 ( $\pm 5$ )	104 ( $\pm 6$ )

The indicated pairs of plasmids were coinjected using a 1:10 molar ratio of enhancer-bearing to enhancer-less plasmid. The numbers of each type of active transcription units observed using oocytes from three different frogs (called a, b, and c) are shown in the next three columns. (Oocytes from different frogs frequently show different total amounts of plasmid transcription but fairly constant relative levels of transcription from co-injected plasmids [e.g., Reeder et al., 1983].) For each gene, 10 representative transcription units, as in Figs. 5 and 6 A, were traced and their nascent transcripts and polymerases were counted (*Pol/TU*). From the unknown lengths of the transcription units, the base pairs per polymerase was calculated (*bp of TU/pol*). Most of the injected plasmid molecules became covered with nucleosomes and showed no nascent transcripts, a result also observed when saturating amounts of template were injected (Trendelenburg and Gurdon, 1978; Bakken et al., 1982a,b). *TU*, transcription unit; *pol*, polymerase with nascent transcript. TU lengths: A6, EA6 (6067 nt); B12, (11960 nt); endogenous (7890 nt).



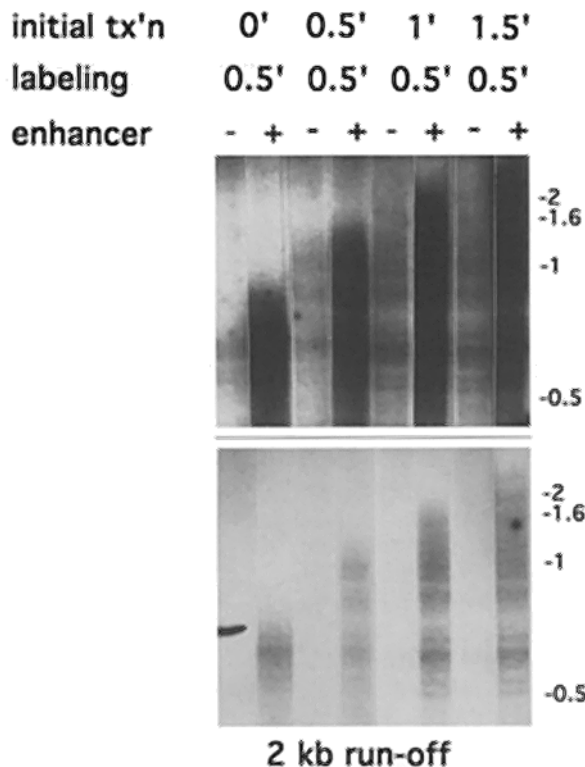


**Figure 7.** Kinetics of rDNA enhancer action. Otherwise equivalent enhancer-less (-) or enhancer-bearing (+) rRNA genes, linearized to generate a 544-nt run-off transcript, were preincubated in L1210 S-100 cell extract for 45 min in the absence of rNTPs (to allow preinitiation complex formation) and then were incubated for 20 min with ATP and CTP (to form the first dinucleotide of the transcript). GTP and UTP were then added for a 0–60 min transcription period, as indicated, after which [ $\alpha^{32}$ P]CTP was added for a 2- or 20-min labeling period, as indicated. The isolated RNA was electrophoretically analyzed. When GTP and UTP are omitted, no run-off transcript is detected.

of enhancer-bearing genes than enhancer-less genes to form actively transcribing complexes.

We have also performed experiments separately injecting oocytes with the same low amounts of either the enhancer-less A6 gene or the enhancer-bearing EA6 gene (data not shown). S1 nuclease analysis showed that the enhancer-bearing promoters generated on average  $\sim 10$ -fold more transcript than the enhancer-less promoters. (This lower value arises in single injection experiments because the enhancer-bearing promoter is subject to *cis* enhancement but the enhancer-less promoter is not subject to *trans* competition [Pape et al., 1989]). The electron microscopic analysis again showed the all-or-none transcription pattern. And again, far fewer active transcription units were observed on the enhancer-less genes than on the enhancer-bearing genes, although the precision of determining the relative transcription levels of singly injected oocytes by microscopy is limited, because the microscopy uses only a few oocytes and individual oocytes exhibit variable transcription capacity. Nonetheless, the data from the various injection protocols at low template concentration are all in concordance.

If enhancer-less genes had instead formed sparsely packed transcription units, these should have been observed in the electron microscopic spreads, for a number of reasons. First, by similarly spreading and assaying oocytes injected with plasmids bearing pol II promoters, we readily observe sparsely packed transcription units, even ones with only one or a few transcripts (Osheim, Y.N., and A. Beyer, unpublished results). These sparse pol II transcription units are observed even when the template plasmids are smaller and their transcription units are much shorter than in the rDNA constructs used in this study. Also, because the vast majority of the plasmids we observe on our grids are inactive ones bearing no transcripts at all, if rDNA plasmids with a few transcripts existed they should have been detected. Additionally, if the observed



**Figure 8.** Transcriptional elongation rate on enhancer-less and enhancer-bearing genes. An experiment like that of Fig. 7 was performed, except that 2-kb enhancer-less (-) or enhancer-bearing (+) run-off templates were used, the initial transcription period in the presence of all four NTPs was 0–1.5 min, as indicated, and the labeling period was 0.5 min. Two exposures of the gel are shown. (The dark diagonal shape on the light exposure is an artifact.) The array of sizes of transcripts represents largely nascent molecules, because upon addition of 300 mM KCl to inhibit further initiation but allow elongation, virtually all these molecules are chased to large size RNAs (data not shown).

amount of total transcript from the enhancer-less gene was generated from sparsely packed transcription units, these sparse transcription units would need to be present in correspondingly greater abundance, and this would have further favored their detection. Yet none such hypothetical sparse pol I transcription units were observed, either under the injection conditions of Figs. 4–6 or when similar experiments were performed using 1/4 the total injected amount of injected promoter (data not shown).

### Analysis of Elongation Rate

The electron micrographic data (Figs. 5 and 6; Table I) indicate that the biochemically assessed effect of the rDNA enhancer—increasing the level of transcript from a *cis*-located vs. a *trans*-located pol I promoter  $\sim 30$ – $50$ -fold (Fig. 4)—can be fully explained by the enhancer not affecting polymerase density but causing  $\sim 20$ – $50$ -fold more *cis*-located promoters to be transcribed (Fig. 1 B). If the enhancer had instead acted by increasing the elongation rate and not the number of active genes, the active transcription units on the enhancer-bearing and enhancer-less gene should be observed at the ratio these genes were mi-

croinjected, yielding  $\sim 1/10$  as many, not  $\sim 3$  times as many, active transcription complexes on the enhancer-bearing genes. Since this was not found (Figs. 5 and 6; Table I), the enhancer can not act primarily by affecting transcriptional elongation rate.

To directly assess whether the rDNA enhancer may also affect transcriptional elongation rate, we turned to an *in vitro* system, where transcriptional stimulation by the repetitive frog and mouse rDNA enhancers can be observed using cell extracts (Pape et al., 1989; Pikaard et al., 1990; Kuhn et al., 1990; Paalman, M.H., D. Snow, J. Wilkinson, and B. Sollner-Webb, manuscript in preparation). With S-100 extract of L1210 cells and low concentration of input mouse rDNA,  $\sim 10$ -fold more transcript is obtained from an enhancer-bearing rDNA promoter than from the same amount of an enhancer-less promoter in a parallel reaction (Paalman, 1995; Paalman, M.H., J. Wilkinson, and B. Sollner-Webb, manuscript in preparation; Fig. 7, *last two lanes*). We first demonstrated that approximately this same level of enhancement is observed throughout the duration of the *in vitro* transcription reaction (Fig. 7). In this experiment, otherwise equivalent enhancer-less or enhancer-bearing templates for a 544 nt run-off transcript were first preincubated in extract lacking rNTPs and then with ATP and CTP to assemble initiated complexes, then a 0–60-min transcription was initiated by addition of rNTPs, and finally [ $\alpha^{32}\text{P}$ ]CTP was added for a 2-min labeling period. Stimulation of the promoter by the adjoining enhancer is similarly seen at short and long times of transcription (Fig. 7). Thus, enhancement is observed at the short reaction times needed to study transcript elongation.

To determine whether the enhancer affects elongation rate, the experimental protocol was modified by using long run-off templates and transcription times that are shorter than are required to synthesize the entire transcript. (It would take  $\sim 1.5$  min to complete a round of transcription on these 2-kb run-off templates at the *in vivo* elongation rate of  $\sim 25$  nt/s.) Again, equimolar amounts of the otherwise equivalent enhancer-less or enhancer-bearing run-off templates were preincubated in S-100 extract, but the initial transcription period was  $\leq 1.5$  min, and the subsequent labeling period was reduced to 0.5 min. Nascent transcripts are detected from both the enhancer-less and enhancer-bearing template, and they grow longer with increasing times of elongation (Fig. 8; two exposures are presented to better observe the transcripts from both genes). Significantly, at each elongation time, the array of transcript lengths are the same from the enhancer-less and the enhancer-bearing template. Therefore, the elongation rates on the enhancer-less and enhancer-bearing genes are equivalent, calculated at  $\sim 20$  nt/s. Yet the enhancer-bearing template generates  $\sim 10$ -fold more transcript than the enhancer-less template (Fig. 8). Enhancement thus does not involve alteration of the transcriptional elongation rate.

## Discussion

The rDNA of most metazoan species examined contains repetitive elements upstream from the gene promoter that are termed enhancers because they augment transcription from a *cis*-located pol I promoter, functioning over dis-

tances and in both orientations (for reviews see Reeder, 1984, 1992; Sollner-Webb and Tower, 1986; Paule, 1994; Jacob, 1995). These enhancers from different metazoan species most likely act by the same mechanism, since frog enhancers function interchangeably with mammalian and plant rDNA enhancers despite a lack of perceptible sequence conservation (Doelling et al., 1993; Kuhn et al., 1990; Pikaard et al., 1990). But how do these, or any, enhancers function to increase transcription from a linked promoter? Two models are that enhancers make individual genes more active or that they activate more genes (Fig. 1).

We have addressed this basic question for the metazoan rDNA enhancer using *Xenopus* oocytes coinjected with marked enhancer-bearing and enhancer-less rRNA genes (Fig. 3). S1 nuclease analysis confirmed that the enhancer exerts an  $\sim 30$ – $50$ -fold relative effect on transcript abundance (Fig. 4), and the morphology and abundance of active transcription units were scored by electron microscopy (Miller and Beatty, 1969) (Fig. 5 and 6). If individual rDNA promoters were made more active by the presence of an enhancer *in cis*, then the enhancer-less promoters could not be fully active, most likely showing transcription units less than maximally packed with nascent transcripts and polymerase (Fig. 1 A). Although low density transcription units can readily be detected from an injected pol II promoter (see above), such were not observed for the injected pol I promoters; the active enhancer-bearing and enhancer-less genes were both fully packed with nascent transcripts, exhibiting identical morphology (Figs. 5 and 6 A) and transcript density (Table I). Furthermore, this conclusion was not affected by the identity of the marked enhancer-bearing and enhancer-less gene (Table I). Instead, the enhancer-bearing rDNA promoters yielded  $\sim 20$ – $50$ -fold more of such active transcription units than the coinjected enhancer-less promoters on a per gene basis (Table I; Fig. 6 B). Thus, the enhancer causes more genes to be actively transcribed (Fig. 1 B).

Two other models could also have explained the fully packed transcription units on the active enhancer-less genes: (1) RNA polymerases could elongate transcription  $\sim 30$ -fold faster on enhancer-bearing genes than those on enhancer-less genes, or (2) the transcripts from enhancer-bearing genes could have an  $\sim 30$ -fold longer half-life than those from enhancer-less genes. However, both of these alternative models imply that enhancer-less and enhancer-bearing genes would be equally likely to generate active transcription units, contrary to the data showing a 30–50-fold difference (Table I). Further dismissing model 1, direct measurement in an enhancer-responsive *in vitro* system confirmed that transcription elongation rate is the same on enhancer-bearing and enhancer-less genes (Fig. 8). Alternate model 2, the enhancer increasing the transcript half-life by  $\sim 30$ -fold, appears inherently unlikely because the transcripts from our enhancer-less and enhancer-bearing genes are identical (Fig. 3). Additionally, finding the same relative accumulation of transcripts from the enhancer-less and enhancer-bearing genes over 2- and 20-min labeling periods (Fig. 7) is contrary to expectation if the enhancer acted by affecting transcript half-life. Thus, the rDNA enhancer does not function by altering transcription elongation rate or transcript half-life. Note that both of these were viable models to explain our earlier

electron microscopic observations on small injected genes (Bakken et al., 1982a,b).

Our data also argue that the enhancer causes a larger fraction of the *cis*-located promoters to be transcriptionally active by favoring formation or use of transcription complexes and not by increasing the longevity of active transcription complexes once they have formed. If the rDNA enhancer allowed a larger number of rounds or a longer period of transcription from each established preinitiation complex, the extent of enhancement on preformed preinitiation complexes should start at a low value and increase with increasing times of transcription. However, the same extent of enhancement is observed immediately after the onset of transcription and after extensive periods of transcription (Fig. 7), including multiple rounds of reinitiation (data not shown).

Yeast rDNA also has an enhancing sequence, but unlike metazoan rDNA enhancers it is nonrepetitive and is at the opposite end of the intergenic spacer (Elion and Warner, 1984). Fivefold transcriptional augmentation by this yeast enhancer can also be observed *in vitro*, and this level is maintained when transcription complexes are allowed to elongate/reinitiate for 10 s and then are supplemented with sufficient sarkosyl to presumably prevent subsequent rounds of initiation (Schultz et al., 1993). Since individual templates should not have initiated five rounds during the 10-s reinitiation period (metazoan rDNA promoters initiate at most 2–3 rounds in that time), the yeast nonrepetitive rDNA enhancer most likely acts to increase the number of transcribing promoters (Schultz et al., 1993). This is consistent with what we find for the repetitive metazoan rDNA enhancer. However, the yeast nonrepetitive rDNA enhancer and the metazoan repetitive rDNA enhancer appear to function differently in other respects, because cleaving off the enhancer segment after preinitiation complex assembly did not impair enhancer function in yeast (Schultz et al., 1993) but eliminated its function in mouse (Paalman, M.H., J. Wilkinson, and B. Sollner-Webb, manuscript in preparation).

Our experiments also underscore the existence of two different modes of rDNA transcriptional initiation in injected oocytes. At subsaturating amounts of injected promoter ( $\leq 2$  fmol per oocyte nucleus; Sollner-Webb and McKnight, 1981), transcription is completely dependent on the normally recognized 140-bp rDNA promoter (Windle and Sollner-Webb, 1986b) and is affected greatly by enhancer sequences (Labhart and Reeder, 1984; Figs. 2 E and 4), being stimulated in *cis* (Pape et al., 1989; Fig. 2 A) and inhibited in *trans* (Labhart and Reeder, 1984; Fig. 2 C). In contrast, when the amount of microinjected promoter is increased to saturating levels ( $\geq 4$  fmol per oocyte nucleus; Sollner-Webb and McKnight, 1981), there is no effect of deleting the binding sites for the normally essential transcription factors SL1 and UBF (Bell et al., 1986; Tower et al., 1986; Pikaard et al., 1989), since the same high levels of +1 initiation by pol I occur when the rDNA segment lacks all sequences upstream of residue -7 (Sollner-Webb et al., 1983; Windle and Sollner-Webb, 1986a). Furthermore, this aberrant mode of initiation is not stimulated by the rDNA enhancer in *cis* (Fig. 2 A) nor inhibited by the enhancer in *trans* (Fig. 2, D and F), suggesting that it does not use the normally essential transcription factor

that is presumed to be titrated by the enhancer repeats at lower template concentration (Labhart and Reeder, 1984). This aberrant mode of initiation is  $>20$ -fold repressed on a per gene basis (and  $>50$ -fold repressed on a per oocyte basis) at subsaturating template concentration, for no +1 initiation has been detected from a deleted rDNA promoter injected at  $\leq 2$  fmol per oocyte (Windle and Sollner-Webb, 1986a; and data not shown). Further affirming that the aberrant mode of transcription is repressed effectively in our experiments (Figs. 4–6), the extent of enhancer stimulation was not increased by reducing the injected promoter concentration another fourfold (see above).

The two modes of rDNA transcription evidently represent substantially different kinds of initiation that use different promoter-factor interactions. Thus, experiments conducted at the high template concentration do not address how the normal rDNA promoter or the rDNA enhancer functions. Because our early electron microscopic analysis of short rRNA genes which retain the promoter and only one copy of the enhancer repeat were performed at 3–6 fmol injected promoter per oocyte nucleus and did not observe any effect of the enhancer (Bakken et al., 1982a,b), the current experiments are novel in studying the effect of the normal promoter and the active rDNA enhancer by electron microscopy.

Might pol II enhancers function in a manner similar to the metazoan pol I enhancer? The vast majority of functional studies on pol II transcriptional activators have been biochemical, averaging out the contribution of many gene copies, so they do not address how the enhancer works on an individual gene basis. The extensive analysis of basal transcription factors interacting with pol II enhancer-binding proteins also has not resolved whether pol II enhancers act by increasing the reinitiation frequency on each active gene or by increasing the number of active genes in a population. Because the enhancer of a single copy gene might be expected to regulate its initiation similarly in individual cells, and because various cellular pol II-transcribed genes with their naturally present enhancers have differing polymerase densities (e.g., Miller and Bakken, 1972; Laird and Chooi, 1976; Foe et al., 1976), it may seem that pol II enhancers would act by increasing reinitiation frequency, unlike the metazoan rDNA enhancer. However, the few available direct cell biological studies on the action of transfected pol II genes indicate that their enhancers increase the chance that the recipient cell will express the gene, rather than increases the gene's expression level in each cell (Weintraub, 1988; White et al., 1992; Walters et al., 1995). We submit that electron microscopic studies on pol II-transcribed genes, such as those performed here on pol I-transcribed genes, could prove highly informative in confirming how various pol II transcriptional enhancers function.

We thank the members of the Sollner-Webb and Beyer laboratories, especially Laura Rusché, for many helpful discussions. We also thank Kathy Jagger for help in preparing the manuscript.

This work was supported by grants from the National Institutes of Health (GM27720) and the National Science Foundation (NSF-DCB-9004860) to B. Sollner-Webb and A. Beyer, respectively.

Received for publication 30 January 1996 and in revised form 13 March 1996.

## References

- Abmayr, S.M., J.L. Workman, and R.G. Roeder. 1988. The pseudorabies immediate early protein stimulates *in vitro* transcription by facilitating TFIID-promoter interactions. *Genes & Dev.* 2:542-553.
- Bakken, A., G. Morgan, B. Sollner-Webb, J. Roan, S. Busby, and R. Reeder. 1982a. Mapping the transcription initiation and termination signal on *Xenopus laevis* ribosomal DNA. *Proc. Natl. Acad. Sci. USA.* 79:56-60.
- Bakken, A., G. Morgan, B. Sollner-Webb, J. Roan, S. Busby, and R. Reeder. 1982b. Mapping the regulators of *Xenopus laevis* ribosomal gene activity. In *Perspectives in Differentiation and Hypertrophy*. Winston Anderson, editor. Elsevier Science Publishing Co. Inc. New York.
- Bell, S., R.M. Learned, H.M. Jantzen, and R. Tjian. 1988. Functional cooperativity between transcription factors UBF1 and SL1 mediates human rRNA synthesis. *Science (Wash. DC).* 241:1192-1197.
- Busby, S., and R. Reeder. 1983. Spacer sequences regulate transcription of ribosomal gene plasmids injected into *Xenopus* embryos. *Cell.* 34:989-996.
- Copenhagen, G., C. Putnam, M. Denton, and C. Pikaard. 1994. The RNA polymerase I transcription factor UBF is a sequence-tolerant HMG-box protein that can recognize structured nucleic acids. *Nucleic Acids Res.* 22:2651-2657.
- DeWinter, R., and T. Moss. 1987. A complex array of sequences enhances ribosomal transcription in *X. laevis*. *J. Mol. Biol.* 196:813-827.
- Doelling, J., R. Gaudino, and C. Pikaard. 1993. Functional analysis of *Arabidopsis thaliana* rRNA gene and spacer promoters *in vivo* and by transient expression. *Proc. Nat. Acad. Sci. USA.* 90:7528-7532.
- Dunaway, M., and P. Droge. 1989. Transactivation of the *Xenopus* rRNA gene promoter by its enhancer. *Nature (Lond.).* 341:657-659.
- Elion, E.A., and J.R. Warner. 1984. The major promoter element of rRNA transcription in yeast lies 2 kb upstream. *Cell.* 39:663-673.
- Foe, V.E., L.E. Wilkinson, and C.D. Laird. 1976. Comparative organization of active transcription units in *Oncopeltus fasciatus*. *Cell.* 9:131-146.
- Foe, V. 1978. Modulation of rRNA synthesis in *Oncopeltus fasciatus*: an electron microscopic study of the relationship between changes in chromatin structure and transcriptional activity. *Cold Spring Harbor Symp. Quant. Biol.* 42:723-740.
- Gerber, H.-P., M. Hagmann, K. Sempel, O. Georgiev, M.A.L. West, Y. Litingtung, W. Schaffner, and J.L. Corden. 1995. RNA polymerase II C-terminal domain required for enhancer-driven transcription. *Nature (Lond.).* 374:660-663.
- Horikoshi, M., M.F. Carey, H. Kakidani, and R.G. Roeder. 1988. Mechanism of action of a yeast activator: direct effect of GAL4 derivatives on mammalian TFIID-promoter interactions. *Cell.* 54:665-669.
- Hu, C.H., B. McStay, S.-W. Jeong, and R.H. Reeder. 1994. xUBF, an RNA polymerase I transcription factor, binds crossover DNA with low sequence specificity. *Mol. Cell. Biol.* 14:2871-2882.
- Jacob, S. 1995. Regulation of ribosomal gene transcription. *Biochem. J.* 306:617-626.
- Kingston, R.E., and M.R. Green. 1994. Modeling eukaryotic transcriptional activation. *Curr. Biol.* 4:325-332.
- Klein, C., and K. Struhl. 1994. Increased recruitment of TATA-binding protein to the promoter by transcriptional activation domains *in vivo*. *Science (Wash. DC).* 266:280-282.
- Kuhn, A., U. Deppert, and I. Grummt. 1990. A 140-base-pair repetitive sequence element in the mouse rRNA gene spacer enhances transcription by RNA polymerase I in a cell-free system. *Proc. Natl. Acad. Sci. USA.* 87:7527-7531.
- Labhart, P., and R.H. Reeder. 1984. Enhancer-like properties of the 60/81 bp elements in the ribosomal gene spacer of *Xenopus laevis*. *Cell.* 37:285-289.
- Labhart, P., and R.H. Reeder. 1985. *Xenopus* ribosomal gene enhancers function when inserted inside the gene they enhance. *Nucleic Acids Res.* 13:8999-9008.
- Laird, C.D., and W.Y. Chooi. 1976. Morphology of transcription units in *Drosophila melanogaster*. *Chromosoma (Berl.).* 58:193-218.
- Learned, R.M., T. Learned, M. Haltiner, and R. Tjian. 1986. Human rDNA transcription is modulated by the coordinate binding of two factors to an upstream control element. *Cell.* 45:847-857.
- Lewin, B. 1990. Commitment and activation at pol II promoters: a tail of protein-protein interactions. *Cell.* 61:1161-1164.
- Miller, O.L., Jr., and B.R. Beatty. 1969. Visualization of nucleolar genes. *Science (Wash. DC).* 164:955-957.
- Miller, O.L., Jr., and A.H. Bakken. 1972. Morphological studies of transcription. *Acta Endocrinol.* 168:1558-1775.
- Miller, O.L., Jr. 1981. The nucleolus, chromosomes, and visualization of genetic activity. *J. Cell Biol.* 91:15s-27s.
- Miller, K.G., and B. Sollner-Webb. 1981. Transcription of mouse rRNA genes by RNA polymerase I: *in vitro* and *in vivo* initiation and processing sites. *Cell.* 27:165-174.
- Mitchelson, K., and T. Moss. 1987. The enhancement of ribosomal transcription by the recycling of RNA polymerase I. *Nucleic Acids Res.* 15:9577-9596.
- Morgan, G., A. Bakken, and R. Reeder. 1982. Transcription of *X. borealis* rRNA genes in nuclei of *X. laevis* oocytes. *Dev. Biol.* 93:471-477.
- Moss, T. 1983. A transcriptional function for the repetitive ribosomal spacer in *Xenopus laevis*. *Nature (Lond.).* 302:223-228.
- Moss, T., and V. Stefanovsky. 1995. Promotion and regulation of ribosomal transcription in eukaryotes by RNA polymerase I. *Nucleic Acids Res.* 50:25-66.
- Mougey, E., M. O'Reilly, Y. Osheim, O.L. Miller, Jr., A. Beyer, and B. Sollner-Webb. 1993. The terminal balls characteristic of eukaryotic rRNA transcription units in chromatin spreads are rRNA processing complexes. *Genes & Dev.* 7:1609-1619.
- Paalman, M.H. 1995. Regulatory element of the mouse rRNA gene. Ph.D. thesis. Johns Hopkins University School of Medicine, Baltimore, MD. 123 pp.
- Paalman, M.H., S.L. Henderson, and B. Sollner-Webb. 1995. Stimulation of the mouse rRNA gene promoter by a distal spacer promoter. *Mol. Cell. Biol.* 15:4648-4656.
- Pape, L., J. Windle, E. Mougey, and B. Sollner-Webb. 1989. The *Xenopus* rDNA 60/81 bp repeats are true 5' enhancers and function at the establishment of the preinitiation complex: analysis *in vivo* and in an enhancer-responsive *in vitro* system. *Mol. Cell. Biol.* 9:5093-5104.
- Paule, M.R. 1994. Transcription of ribosomal RNA by eukaryotic RNA polymerase I. In *Transcription: Mechanisms and Regulation*. R.C. Conaway and J.W. Conaway, editors. Raven Press, Ltd., New York. 83-106.
- Pikaard, C. 1994. Ribosomal gene promoter domains can function as artificial enhancers of RNA polymerase I transcription. *Proc. Natl. Acad. Sci. USA.* 91:464-468.
- Pikaard, C., and R.H. Reeder. 1988. Sequence elements essential for function of the *Xenopus laevis* ribosomal DNA enhancers. *Mol. Cell. Biol.* 8:4282-4288.
- Pikaard, C., B. McStay, M.C. Schultz, S.P. Bell, and R.H. Reeder. 1989. The *Xenopus* ribosomal gene enhancers bind an essential polymerase I transcription factor, xUBF. *Genes & Dev.* 3:1779-1788.
- Pikaard, C., L. Pape, S. Henderson, K. Ryan, M. Paalman, M. Lopata, R. Reeder, and B. Sollner-Webb. 1990. Enhancers for RNA polymerase I in mouse ribosomal DNA. *Mol. Cell. Biol.* 10:4816-4825.
- Ptashne, M., and A.A.F. Gann. 1990. Activators and targets. *Nature (Lond.).* 346:329-331.
- Reeder, R.H., J.G. Roan, and M. Dunaway. 1983. Spacer regulation of *Xenopus* ribosomal gene transcription: competition in oocytes. *Cell.* 35:449-456.
- Reeder, R.H. 1984. Enhancers and ribosomal gene spacers. *Cell.* 38:349-351.
- Reeder, R.H. 1992. Regulation of transcription by RNA polymerase I. In *Transcriptional Regulation*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 315-347.
- Roberts, S.G.E., I. Ha, E. Maldonado, D. Reinberg, and M.R. Green. 1993. Interaction between an acidic activator and transcription factor TFIIB is required for transcriptional activation. *Nature (Lond.).* 363:741-744.
- Schultz, M.C., S.Y. Choe, and R.H. Reeder. 1993. *In vitro* definition of the yeast RNA polymerase I enhancer. *Mol. Cell. Biol.* 13:2644-2654.
- Sollner-Webb, B., and S. McKnight. 1981. Accurate transcription of cloned *Xenopus* ribosomal RNA genes. *Nucleic Acids Res.* 10:3391-3405.
- Sollner-Webb, B., and J. Tower. 1986. Transcription of cloned eukaryotic ribosomal RNA genes. *Annu. Rev. Biochem.* 55:801-830.
- Sollner-Webb, B., J. Wilkinson, J. Roan, and R. Reeder. 1983. Nested control regions promote *Xenopus* ribosomal RNA synthesis by RNA polymerase I. *Cell.* 35:199-206.
- Tower, J., V. Culotta, and B. Sollner-Webb. 1986. The factors and nucleotide sequences that direct rDNA transcription and their relationship to the stable transcription complex. *Mol. Cell. Biol.* 6:3451-3462.
- Trendelenburg, M.F., and J. Gurdon. 1978. Transcription of cloned *Xenopus* ribosomal genes visualized after injection into oocyte nuclei. *Nature (Lond.).* 276:292-294.
- Walters, M.C., S. Fiering, J. Eidemiller, W. Magis, M. Groudine, and D.I. Martin. 1995. Enhancers increase the probability but not the level of gene expression. *Proc. Natl. Acad. Sci. USA.* 92:7125-7129.
- Weintraub, H. 1988. Formation of stable transcription complexes as assayed by analysis of individual templates. *Proc. Natl. Acad. Sci. USA.* 85:5819-5823.
- White, M., M. Masuko, L. Amet, G. Elliott, M. Braddock, A. Kingsman, and S. Kingsman. 1992. Real-time analysis of the transcriptional regulation of HIV and hCMV promoters in single mammalian cells. *J. Cell Sci.* 108:441-455.
- Windle, J., and B. Sollner-Webb. 1986a. Upstream domains of the frog rDNA promoter are revealed in microinjected oocytes. *Mol. Cell. Biol.* 6:1228-1234.
- Windle, J., and B. Sollner-Webb. 1986b. Two distinct and precisely positioned domains promote transcription of *Xenopus* rRNA genes: analysis with linker scanning mutants. *Mol. Cell. Biol.* 6:4585-4593.
- Workman, J.L., S.M. Abmayr, W.A. Cromlish, and R.G. Roeder. 1988. Transcriptional regulation by the immediate early protein of pseudorabies virus during *in vitro* nucleosome assembly. *Cell.* 55:211-219.
- Xiao, H., A. Pearson, B. Coulombe, R. Truant, S. Zhang, J.L. Regier, S.J. Triezenberg, D. Reinberg, O. Flores, C.J. Ingles et al. 1994. Binding of basal transcription factor TFIIF to the acidic activation domains of VP16 and p53. *Mol. Cell. Biol.* 14:7013-7024.
- Zawel, L., and D. Reinberg. 1993. Initiation of transcription by RNA polymerase II: a multi-step process. *Prog. Nucleic Acid Res. Mol. Biol.* 44:67-108.
- Zawel, L., K.P. Kumar, and D. Reinberg. 1995. Recycling of the general transcription factors during RNA polymerase II transcription. *Genes & Dev.* 9:1479-1490.