Expression of ribosomal insertion in Drosophila: sensitivity to intercalating drugs

Igor B. Dawid and Martha L. Rebbert

Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892, USA

Received 14 November 1985; Accepted 14 January 1986

ABSTRACT

Ribosomal insertions in Drosophila are transcribed at very low levels. The abundance of the most prominent 0.8 kb type 1 insertion transcript increased up to 60-fold when cultured cells were exposed to the DNA intercalating drug chloroquine. After injection of insertion-containing rDNA in circular form into Xenopus laevis oocytes an apparently identical 0.8 kb insertion transcript was synthesized, and its accumulation was stimulated several fold by coinjection of chloroquine or ethidium bromide. We suggest that ribosomal insertions are assembled in a chromatin conformation that lacks unconstrained torsional stress, accounting for the inactivity of these DNA regions; introduction of stress by intercalation results in activation of transcription from the insertion sequences.

INTRODUCTION

In Drosophila, many of the ribosomal DNA repeating units are interrupted in the 28S RNA sequence by noncoding DNA segments called ribosomal insertions. In D. melanogaster ribosomal insertions occur in two unrelated sequence types, and each type is present in multiple length classes (reviewed in ref. 1). The ribosomal repeats carrying insertions are transcribed at a very low level only, precluding their contribution to rRNA synthesis in the cell (2). The biological inactivity of interrupted rDNA repeats has been demonstrated clearly in D. hydei by showing that such interrupted units have no phenotypic consequence (3). Thus, all rPNA transcription appears to be supported by the uninterrupted genes, and no splicing has been demonstrated in Drosophila rRNA biogenesis. Nevertheless, ribosomal insertions are transcribed at low levels yielding some polydispersed and certain discrete transcription products (2). The phenomena described above raise the question of the mechanism of differential regulation of transcription of rRNA genes. In D. melanogaster the interrupted and uninterrupted rDNA repeats are indistinguishable in sequence in their promoter regions (4), and promoters derived from interrupted genes are active in vitro (5). Mechanisms not depending on
promoter sequence alone must be responsible for the paucity of insertion transcripts in Drosophila cells.

Among the mechanisms believed to control gene activity in eukaryotes DNA supercoiling has recently achieved renewed attention. While it is generally believed that most DNA in the nucleus does not carry unconstrained torsional stress, evidence has emerged in several systems to indicate that such unconstrained stress is present in certain transcribed gene regions (6-12). This evidence suggests that active genes may be supercoiled since relieving of stress, for example by novobiocin inhibition of topoisomerase II, interferes with the assembly or maintenance of an active chromatin configuration (8,11). The most relevant case in the present context is the work of Pruitt and Reeder (6) who showed that the normally silent "spacer promoters" in rDNA of Xenopus oocytes become activated by the injection of moderate doses of the intercalating drug ethidium bromide (EB). This work suggests that the spacer promoters, unlike the ribosomal gene promoter, are naturally in a constrained and thus inactive configuration, but can be rendered torsionally stressed and active by changes in DNA supercoiling. To investigate whether the state of supercoiling of interrupted rDNA repeats in Drosophila is relevant to their transcriptional inactivity we tested whether intercalating drugs affect the level of transcription from ribosomal insertions in Drosophila cells and in a heterologous transcription system.

MATERIALS AND METHODS

The rRNA clone C53, containing a 1 kb type 1 insertion, has been described (13, 14). Minigene construction and subcloning followed general procedures outlined by Maniatis et al. (15).

D. melanogaster Schneider II cells were grown under the conditions described (16). The cells were exposed to chloroquine at the mid-log period of growth, adding the drug to cultures containing between 0.5 and 1x10⁶ cells/ml. Chloroquine intercalates into DNA (17) and has been used previously to affect transcription in cultured cells (6). DNA transfection into Schneider cells for transient expression was carried out as described previously (16).

X. laevis oocytes were freed of their follicle cells with collagenase (18), and about 20 nl DNA solution with or without drug was injected into their nuclei as described (19).

RNA was prepared as described from Drosophila cells (2), and from oocytes (20). RNA was separated by gel electrophoresis in methylmercury hydroxide agarose gels (21). RNA was transferred from gels or dotted onto
Figure 1. Map of minigene mC53. The minigene was derived from C53, a rDNA repeat containing a 1 kb type 1 insertion (13,14). As shown in the expanded portion of the figure, a segment of rDNA containing the upstream spacer and the first 32 bp of the rRNA transcription unit was fused to a site in the 28S rRNA region 1 kb upstream of the insertion. Beyond the fusion point the minigene continues through the insertion, the 3' terminus of the 28S rRNA region and the following spacer. Relevant restriction sites are shown as follows: N, NruI; T, TaqI; B, BamHI; Bg, BglII. The insertion probes are shown below the map, including the BamHI fragment corresponding to most of the 1 kb insertion, and the BamHI/BglII and the BglII/BamHI probes which correspond to the left and right ends of the insertion, respectively (14). In the lower part of the figure the probe and the protected fragment in the S1 experiment of Figure 3 are shown.

nylon membranes and hybridized with nick-translated DNA probes as described by Church and Gilbert (22). The insertion probe used has been subcloned (2) to reliably purify it from rRNA sequences.

RESULTS

Stimulation of Insertion Transcription by Chloroquine

If interrupted rDNA repeats are transcriptionally inactive because they are assembled into a chromatin conformation that lacks torsional stress it should be possible to stimulate transcription from the insertions with the aid of intercalating drugs. Drosophila cells in culture were exposed to different concentrations of chloroquine for different times, the RNA extracted, and hybridized with a restriction fragment that represents the type 1 ribosomal insertion.

Figure 1 shows a map of an interrupted ribosomal gene carrying a 1 kb insertion; this map is that of a minigene construct that we shall return to later in this work. The 1 kb insertion is identical in sequence to the right (downstream) end of the more common 5 kb insertion (23) and the subcloned BamHI fragment used as probe (indicated in Fig. 1) shares homology with all size classes of type 1 sequences.

Exposure of cultured cells to chloroquine results in a dramatic increase in the abundance of insertion transcripts homologous to the BamHI fragment.
Figure 2. Chloroquine stimulation of insertion transcript.
A. Dot blot analysis of RNA extracted from Schneider cells exposed to various concentrations of chloroquine for 1 or 2 days. Two separate experiments were done for the 1 day exposure, one from 2 to 50 mM and the other from 50 to 250 mM chloroquine, as indicated in the figure. At 100 and 250 mM drug concentration the cells were clearly not healthy. Each dot contained 1 µg total cell RNA. The dot labeled "Cont 12x" shows a 12 times longer exposure of the spot immediately above it containing control RNA. B. Gel blot of RNA from cells treated with 25 mM chloroquine for 2 days (lane 2), and control cells (lanes 1,3). Each lane contained 2.5 µg total RNA. The position of 1 and 2 kb bands is indicated. All filters were hybridized with nick translated insertion BamHI fragment.

as shown in Figure 2. This increase can be seen after a 4 hr exposure to the drug (not shown) but is more evident after 1 or 2 days. While 2 mM chloroquine gives a distinct effect the maximal increase in insertion transcripts is seen at 50 mM of the drug in 1 day or 25 mM in 2 days (Fig. 2A). Under these conditions the abundance of insertion transcript increases by up to 60-fold. Concentrations of 100 mM or higher lead to lower transcript abundances presumably because the cells are quite sick under these conditions. However, exposures at the maximal stimulatory level are not permanently toxic: upon removal of the drug the cells continue to grow at normal rate.

The major transcript derived from the type 1 insertion is represented by a RNA molecule of 0.8 kb that is found predominantly in the cytoplasm of
embryos or cultured cells (2). Chloroquine stimulation results in increased abundance of this 0.8 kb RNA molecule, as shown in the Figure 2B.

The same RNA preparations as those shown in Figure 2 were also hybridized to an 1 kb EcoRI fragment derived from type 2 ribosomal insertions (24); no change in the abundance of transcripts could be detected. Chloroquine treatment of these Drosophila cells also did not increase the level of transcription from the ribosomal spacer, as assayed by hybridization with the 250 bp AluI fragment which represents the majority of spacer sequences (25). Furthermore, total RNA synthesis as estimated by the incorporation of uridine into precipitable form, did not differ substantially between control and chloroquine-treated cells.

Transcription of Ribosomal Insertion in Transfected Cells and in Frog Oocytes

We wished to study the transcription of the type 1 insertion in reconstructed systems; for this purpose the minigene shown in Figure 1 was constructed. About 4.7 kb of rRNA coding region was removed from a gene that carries a 1 kb insertion. The remaining sequences include a long upstream spacer, the initiation site of transcription, and the first 32 bp of pre-rRNA fused to a point in the 28S RNA region 1 kb upstream of the site of insertion. The experiments of Kohorn and Rae (5,26,27) showed that constructs containing the upstream spacer and 4 bp of gene region were competent in accurate initiation in vitro. These results suggested that the minigene construct should initiate transcription faithfully in vivo.

As shown in Figure 3 this is the case. Cultured cells were transfected with mC53 DNA by a calcium phosphate procedure (15), cultured for 2 days and the RNA assayed by an S1 procedure which is capable of determining the initiation site of the minigene transcript in the presence of excess indigenous pre-rRNA. As shown in Figure 1 the minigene-derived probe is not continuously homologous to indigenous rRNA; indigenous rRNA is expected to protect the probe from the labeled end to the fusion point whereas minigene transcript should protect it from the labeled end to the transcription start. The results show that the minigene initiates transcription at the same site demonstrated earlier for initiation of resident rRNA genes (4). This result shows that the presence of the insertion in the molecule does not in itself prevent transcription from the rDNA promoter within living Drosophila cells.

Minigene constructs and unaltered rRNA genes from Drosophila were injected into Xenopus oocytes, but in no case was initiation from the rDNA promoter observed. This result is expected on the basis of observations by others that polymerase I transcription of rDNA is species specific (28,29).
Figure 3. Initiation of transcription from rDNA construct in Schneider cells. The minigene mC53 (Figure 1) was transfected into cells with the aid of calcium phosphate. After 3 days of culture the RNA was extracted and analyzed by S1 mapping with a probe labeled at a TaqI site 65 nt downstream from the normal rRNA initiation site (see Figure 1). Lanes 1, 2 contained RNA from transfected cells, lane 3 contained RNA from control cells; the subsequent lanes are sequencing ladders (32) derived from the labeled probe. The line indicates the nucleotide corresponding to the protected fragment taking into account the shift between S1 protected and chemically cleaved fragments (33). The result indicates initiation at the sequence (coding strand) 5'TCCATCCG..., the same as found previously for initiation on the resident rDNA (4).

In contrast, insertion transcripts were seen after injection of rDNA constructs into frog oocytes as well as after transfection into Drosophila cultured cells. In the experiment shown in Figure 4 both a complete
Figure 1. Insertion transcripts in transfected cells and in frog oocytes, as visualized by RNA gel blotting and hybridization with the insertion BamHI fragment. The complete rDNA repeat C53 was used in lanes 1-3, and the minigene mC53 in lanes 4-6. Lanes 1,4: rDNA (200 µg/ml) injected into frog oocytes and incubated overnight; lanes 2,5: rDNA and α-amanitine (10 µg/ml) injected into oocytes; lanes 3,6: Schneider cells transfected with rDNA. The position of 1 and 2 kb bands is indicated.

interrupted rDNA repeat, C53, and the minigene mC53 (Fig. 1) were utilized. Transfection into Drosophila cells (lanes 3,6) lead to an increase in the abundance of the 0.8 kb insertion transcript; control cells contain such an RNA molecule (Figure 2) but do not give a visible band at the exposure time of Figure 4. Injection of either C53 or mC53 into the nucleus of frog oocytes lead to vigorous production of a 0.8 kb RNA molecule that comigrates with the insertion transcript from Drosophila cells (lanes 1,4). While we have not
established unequivocally that the transcripts from the oocyte and from Drosophila are identical this appears likely because of their size and the fact that both are homologous to the downstream end of the insertion (the Bg/B probe of Figure 1) but not the upstream end of the insertion (the B/Bg probe). Figure 4 (lanes 2,5) also shows that injection of α-amanitine into the frog oocyte completely prevents accumulation of insertion transcripts, indicating that polymerase II, rather than polymerase I, is responsible for synthesizing this RNA. We do not know which polymerase transcribes the resident insertions in Drosophila, but the fact that an apparently identical RNA is produced in the frog oocyte by polymerase II raises the possibility that the same enzyme is responsible for the production of the relatively rare RNA molecules derived from type 1 insertions in various Drosophila cells.
Intercalating Drugs Stimulate Insertion Transcription in Frog Oocytes

The synthesis in frog oocytes of an RNA molecule of identical size to the major type 1 transcript in Drosophila cells makes the oocyte a useful model system and allowed the study of the effect of ethidium bromide (EB) on insertion transcription. EB addition to cultured Drosophila cells had no effect, presumably because the drug is not efficiently incorporated into the cells. Figure 5 shows an experiment in which the insertion-containing minigene mC53 was injected into oocyte nuclei together with different concentrations of chloroquine or EB. Chloroquine injected at 100 mM (yielding an intranuclear concentration of about 30 mM) stimulated the accumulation of the 0.8 kb insertion transcript 5- to 10-fold. Injection of 200 mM chloroquine is apparently toxic. An about 10-fold stimulation of insertion transcript accumulation was detected after injection of EB between 25 and 250 μg/ml. These results are similar to the concentration dependence of the stimulation of the spacer promoter of Xenopus rDNA observed by Pruitt and Reeder (6), which showed maximal stimulation between 20 and 500 μg/ml EB. The results of Figure 5 suggest that both chloroquine and EB act by way of their DNA intercalating ability since this activity is common to the two drugs.

DISCUSSION

The experiments reported in this paper show that transcripts derived from type 1 ribosomal insertions in Drosophila, which normally are quite rare, accumulate to substantially higher levels as a result of treatment of cultured cells with chloroquine. Similarly, in the heterologous system of Xenopus oocytes injected with Drosophila rDNA, the level of insertion transcripts is stimulated by chloroquine and by ethidium bromide. These observations provide evidence that the stimulatory effect is due to the DNA intercalating activity of these drugs. This conclusion, together with the work of others on the relationship between gene activity and unconstrained stress in DNA, leads to the suggestion that interrupted rDNA repeats may be in a different chromatin configuration than uninterrupted ribosomal genes which are responsible for the production of rRNA in the cell. The resident rDNA in Drosophila may thus be organized in chromatin in such a way that only the promoter and surrounding regions of the uninterrupted genes are exposed to unconstrained torsional stress, while the interrupted genes are assembled into unstressed, "static" chromatin (8,9). Changes in supercoiling introduced into the interrupted rDNA chromatin by intercalating drugs would then
lead to a substantially increased transcriptional activity and to higher levels of insertion transcripts.

The behavior of Drosophila rDNA injected into Xenopus oocytes supports the above interpretation. The activity of any gene injected into the oocyte is dependent on supercoiling since linear DNAs are much less active \( (10,3^\circ) \). This is also true for Drosophila rDNA: we have injected mC53 linearized at different positions of the molecule and failed to find any transcripts. In the oocyte circular DNA is assembled into chromatin; the nature of the chromatin depends on the sequence of the injected DNA and on the presence of specific transcription factors. Worcel and his colleagues \( (8,9,31) \) have shown that circular DNA containing the 5S RNA gene may be assembled into active or dynamic chromatin if the transcription factor TFIIIA is present in sufficient concentration; otherwise the DNA is assembled into static chromatin. We may assume that Drosophila rDNA would be assembled into static chromatin in the oocyte since a specific transcription factor is not likely to be present. Since RNA is transcribed, however, this static chromatin still has some activity, or alternately, some rDNA molecules are assembled into dynamic chromatin with the aid of non-specific transcription factors present in the oocyte. The activation of transcription of the injected rDNA by chloroquine or EB would then be interpreted as resulting from the introduction of torsional stress into the static rDNA minichromosomes so that they take on some properties of dynamic chromatin.

In summary, these observations suggest that interrupted rDNA repeats in Drosophila are in a chromatin conformation that lacks unconstrained torsional stress; this structure may be responsible, at least in part, for the low transcriptional activity of these DNA sequences. Introduction of stress by means of intercalating drugs relieves this limitation and consequently leads to an activation of insertion transcription.

ACKNOWLEDGMENT

We thank Ronald Reeder for his suggestions which stimulated much of this work and for making unpublished information available.

REFERENCES
