Proc. Natl. Acad. Sci. USA
Vol. 76, No. 6, pp. 2590–2594, June 1979
Biochemistry

Delimitation of a promoter for RNA polymerase III by means of a functional test

(oocyte injection/cloned DNA/tRNA$_{1}^{Met}$ biosynthesis/surrogate genetics)

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Communicated by A. Frey-Wyssling, March 5, 1979

ABSTRACT A *Xenopus laevis* DNA segment containing the structural gene for tRNA$_{1}^{Met}$ and 22 base pairs at the 5′ side of the gene is active in tRNA production. The DNA segment was ligated to sea urchin histone DNA and was also inserted into plasmid pCR1. Both recombinant DNAs were shown to produce mature tRNA$_{1}^{Met}$ at a high rate when injected into centrifuged *Xenopus* oocytes.

The results obtained from gene cloning and DNA sequence analyses have revealed many predicted, and some very unexpected, features of eukaryotic gene organization. It has also become evident that, as yet, regulatory signals cannot be identified simply by inspection of the DNA sequences alone. However, it should be possible to identify such regulatory sequences by a functional test if the structural genes they control can be isolated and brought to faithful expression. This can be achieved most simply by injecting cloned gene units, in their natural form or after sequence manipulation, into the nucleus of *Xenopus* oocytes. In this paper we have been able to delimit a promoter region for RNA polymerase III controlling the tRNA$_{1}^{Met}$ gene by means of a functional test using the centrifuged oocyte injection technique (1).

The cloned DNA containing genes coding for tRNA$_{1}^{Met}$ (tDNA$_{1}^{Met}$) of *X. laevis* has been the subject of comprehensive restriction and sequence analyses (refs. 2 and 3; unpublished data). The cloned tDNA fragment (t210) used in these experiments is 3.18 kilobases long (2) and, besides much spacer DNA, contains two genes coding for tRNA$_{1}^{Met}$ and at least five other tRNA genes, one of which codes for a tRNA^{Leu} species (ref. 3; unpublished data). We have reported previously that, after nuclear injection of the cloned t210, tRNAs are produced at a high rate from the injected template, with as much as 70% of all cellular RNA synthesis being dedicated to the production of these tRNAs (1, 4). Injection experiments with DNA subfragments suggested that the sequences important for the initiation of transcription might be found by the simple procedure of trimming the sequence flanking the genes to the point at which tRNA production was abolished (4). By dissection of this cloned tDNA we have now been able to narrow down the sequence still capable of producing tRNA$_{1}^{Met}$ and have found this to be a short *Hin*fI DNA restriction fragment containing, besides the gene itself and some trailing sequences, only 22 base pairs of DNA adjacent to the 5′ end of the structural gene.

MATERIALS AND METHODS

Enzymes and Reagents. T4 DNA ligase and restriction endonucleases *Alu* I, *Hin*dIII, *Hin*fI, and *Hpa* II were obtained from New England BioLabs; *Eco*RI and Klenow DNA polymerase (5) were purchased from Boehringer Mannheim. *Eco*RI linkers were the gift of W. Schaffner and were originally obtained from Collaborative Research (Waltham, MA).

Preparation of tDNA Fragments. Three milligrams of pBR322-t210 DNA was digested with *Hin*dIII, extracted with phenol, and precipitated with ethanol. The DNA digestion products, dissolved in 10 mM Tris-HCl/1 mM EDTA at pH 8, were mixed at 50°C with an equal volume of 1% agarose solution poured onto a cylindrical 1% agarose gel (diameter, 8 cm) made up in 50 mM glycine/NaOH buffer containing 1 mM EDTA at pH 9.2. After gel electrophoresis overnight at 1 V/cm, the gel was sliced into quadrants and stained with ethidium bromide, and the tDNA band was extracted. The tDNA was purified by CsCl density centrifugation before digestion with either *Alu* I or *Hpa* II. The largest fragments (fragments A) were separated from the other cleavage products on cylindrical gels (diameter, 3 cm) as described above. The *Hpa* II fragment A was treated with bacterial alkaline phosphatase (6) and cleaved with *Hin*fI. The fragments a, b, c, and d (see Fig. 1 and Fig. 2, slot a) were purified by gel electrophoresis followed by DEAE-cellulose chromatography (Fig. 2, slot a) and used in ligation experiments. Ligation of the *Hin*fI fragments was carried out in a final volume of 10–25 μl containing microgram quantities of the DNA to be ligated together with 1 unit of T4 DNA ligase in 50 mM Tris-HCl, pH 7.5/10 mM $MgCl_2$/10 mM dithiothreitol/0.07 mM rATP. After a 3-hr incubation at 15°C, the DNA was diluted to 0.1 ml with 2% sodium acetate (pH 7.5) and extracted with phenol. The amount of DNA and the extent of ligation were determined by gel electrophoresis in comparison with known amounts of DNA molecular weight standards. Approximately equimolar amounts of ligation products and *Alu* I fragment A were mixed together, precipitated with ethanol, and dissolved in injection buffer (7). In some experiments, histone DNA was added as carrier.

Construction of Recombinant pCR1-tmetA1. The 5′-overhanging ends of *Hin*fI fragment c were filled in by incubation of 1 pmol of DNA with 1 unit of Klenow DNA polymerase (5) in 20 μl of 50 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 20 mM dithiothreitol, 1 mM rATP, and 200 μM each of dATP, dCTP, dGTP, and dTTP. Incubation was for 10 min at 15°C. After inactivation of the enzyme by a 5-min incubation at 65°C, 25 pmol of phosphorylated (6) *Eco*RI linkers were added and ligation was performed with 0.5 unit of T4 DNA ligase for 12 hr at 15°C. The enzyme was then inactivated by heating. The excess linker fragments were removed by digestion of the incubation mixture with 50–100 units of *Eco*RI. After extraction of the proteins with phenol/chloroform, 1:1 (vol/vol) and three times with chloroform, the aqueous phase was precipitated with ethanol and the DNA was used for integration into plasmid pCR1 that had been made linear with *Eco*RI and treated with

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Abbreviation: tDNA, DNA containing genes coding for tRNA.

bacterial alkaline phosphatase. The fragments were ligated with sticky ends in 50 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$/10 mM dithiothreitol/0.07 mM rATP. After transfection of $CaCl_2$-treated *Escherichia coli* HB 101 cells, recombinant colonies were selected by hybridization with nick-translated t210. The containment conditions used for the construction and amplification of the recombinant plasmids were those specified by the National Institutes of Health Guidelines (P2, EK1).

DNA Injection and RNA Analysis. A simplified oocyte injection technique developed in our laboratory (1) was used throughout these experiments. *Alu* I fragment A and manipulated *Hpa* II fragment A, together with 0.1 μCi of [α-^{32}P]GTP (Amersham; 1 Ci = 3.7×10^{10} becquerels) were injected into oocytes at a molar concentration equivalent to 1 ng of t210 DNA per oocyte. The incubation time was usually 3–4 hr (3–16 hr for pCR1-tmetA1 injection experiments). The oocytes were homogenized and the RNA was extracted (4). The RNA was passed over a Sephadex G50 column to remove unincorporated labeled GTP and fractionated on a 5–20% sucrose gradient. The 4S region was collected and the RNA was recovered by ethanol precipitation for polyacrylamide gel analysis.

One-dimensional 10% polyacrylamide gels containing 7 M urea ($0.05 \times 25 \times 25$ cm) were electrophoresed at 10 W for 3.5 hr at 4°C with Tris borate/EDTA buffer (80 mM Tris borate/1 mM EDTA, pH 8.3). For two-dimensional analyses (8), gels contained 4 M urea and Tris borate/EDTA. The first dimension 10% gel ($0.2 \times 25 \times 37$ cm) was overlaid with a 4% stacking gel ($0.2 \times 25 \times 3$ cm) containing 4 M urea and Tris borate/EDTA adjusted to pH 6.7 with HCl. Electrophoresis was at 400 V for 23 hr at 4°C. The second dimension 20% gel ($0.2 \times 25 \times 25$ cm) was electrophoresed at 400 V for 43 hr at 4°C. Running buffer for all gels was Tris borate/EDTA, pH 8.3, without urea.

RNA was electrophoretically eluted from gels and digested with RNase T1. The oligonucleotides were separated by iontophoresis on cellulose acetate strips at pH 3.5 followed by homochromatography on polyethyleneimine thin-layer plates (9, 10).

RESULTS

Experimental Design. The tDNA (t210) used in the experiments reported here was initially cloned and amplified in a λ vector (2). The polarity and locations of the $tRNA_1^{Met}$ and $tRNA^{Leu}$ genes contained in this clone are shown in Fig. 1, together with the relevant restriction maps and DNA sequence information (refs. 2 and 3; unpublished data). To improve DNA yields, we recovered the entire 3.18-kb tDNA fragment from the λ vector and inserted it into pBR322 as a trimer via *Hin*dIII termini. After *Hin*dIII restriction of the recombinant DNA, the tDNA was isolated by preparative agarose gel electrophoresis and was digested with either *Alu* I or *Hpa* II. The largest fragments were then prepared from both of these digests (fragments A). In this way, tDNA segments containing the $tRNA^{Leu}$ gene or the "first" or "A" $tRNA_1^{Met}$ gene, together with some flanking sequences, were obtained (Figs. 1 and 2). For the manipulation of the DNA sequences adjacent to the $tRNA_1^{Met}$ gene A, the serendipitous *Hin*fI restriction sites stragically placed around the gene were used to delimit sequences essential for transcription. The *Hin*fI fragment c, containing the structural gene, was extended by ligation with fragments a and b (Figs. 1 and 2), and the biological activity of each reconstructed tDNA was tested in oocytes. *Alu* I fragment A was always coinjected with the *Hpa* II fragment A or its derivatives, and $tRNA_1^{Met}$ production was compared to $tRNA^{Leu}$ synthesis, which served as an internal control.

Small DNA Subfragments A Are Still Capable of Producing $tRNA^{Leu}$ and $tRNA_1^{Met}$. In a first series of experiments we tested fragments A of both the *Alu* I and the *Hpa* II digests for their suitability as templates when injected into oocyte nuclei. Centrifuged oocytes were divided into three groups: the first group was injected with [α-^{32}P]GTP alone, the second with

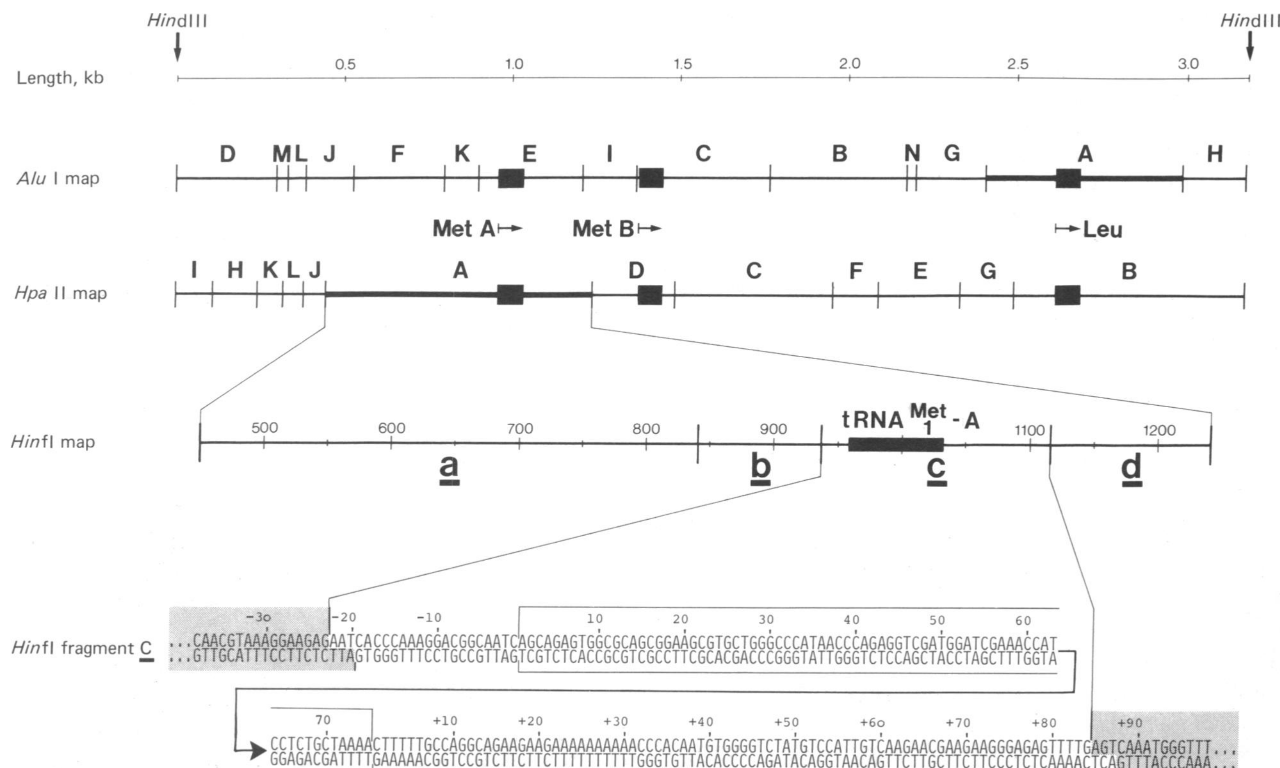

FIG. 1. Structure of the cloned 3.18-kilobase tDNA fragment (t210) of *X. laevis*. Relevant restriction maps, gene locations and polarity, and the nucleotide sequences in and around the gene A coding for $tRNA_1^{Met}$ are indicated (refs. 2 and 3; unpublished data). The sequences encoding the mature $tRNA_1^{Met}$ are enclosed in a box. Sequences adjacent to the 5′ end of the structural gene are indicated by (−); 3′ flanking sequences are indicated by (+). kb, Kilobase.

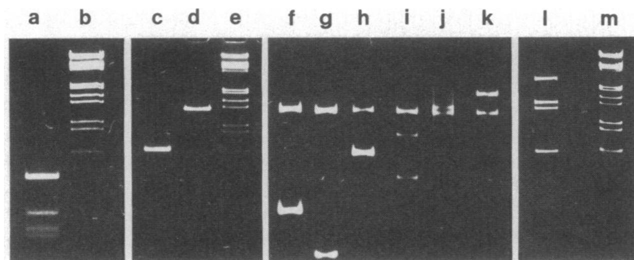

FIG. 2. Restriction fragments of t210 and their ligation products. Electrophoresis was through gels of 1.5% agarose (slots a–e, l, and m) or 5% polyacrylamide (slots f–k). Slot a, *Hin*fI digestion products of the *Hpa* II fragment A; four DNA fragments were obtained, in order of descending molecular weights a, c, d, b. Slot b, λ marker digested with *Eco*RI and *Hin*dIII. Slot c, *Alu* I fragment A. Slot d, h22 sea urchin histone DNA fragment containing one terminal *Hin*dIII site and a *Hin*fI sequence appropriate for ligation to the 5′ end of fragment c (see Fig. 1). Slot e, λ marker. Slot f, *Alu* I fragment A (more slowly moving component) and fragment c of Fig. 1 (faster moving component). Slot g, *Alu* I fragment A and fragment b of Fig. 1. Slot h, *Alu* I fragment A and fragment a of Fig. 1. Slot i, *Alu* I fragment A and ligated fragments b-c and c-b-c. Slot j, *Alu* I fragment A and ligated fragments a-b-c. Slot k, *Alu* I fragment A and *Hpa* II fragment A. Slot l, *Alu* I fragment A and ligation products h22-fragment c, h22-fragment c produced by *Hin*dIII ligation, and a minor portion of higher order copolymers. Slot m, λ marker.

labeled GTP and *Alu* I fragment A, and the third with labeled GTP and both *Alu* I and *Hpa* II fragments A. After a 3-hr incubation, total RNA was prepared and the RNA was fractionated on a sucrose gradient. The 4S region was recovered and the labeled RNAs were fractionated by polyacrylamide gel electrophoresis (Figs. 3 and 4).

Somatic 4S RNA prepared from ^{32}P-labeled *X. laevis* tissue culture cells can be resolved, as expected, into a large number of components of distinct mobilities by two-dimensional gel electrophoresis (Fig. 3*a*). Oocytes at stage V and VI synthesize much ribosomal RNA but little 4S and 5S RNA. Hence, the autoradiogram of the RNA synthesized by oocytes injected with [α-^{32}P]GTP alone shows only minor incorporation into tRNA species (Fig. 3*b*). However, after injection of the *Alu* I fragment A, one heavily-labeled component and one minor spot clearly dominate the endogenous 4S RNA background (Fig. 3*c*). After simultaneous injection of *Alu* I and *Hpa* II fragments A, the same two spots are found, together with an additional 4S RNA that migrates faster in both dimensions (Figs. 3*d* and 4, lane a). To identify these RNAs unequivocally, we isolated each component from one-dimensional gels and analyzed them by RNase T1 fingerprinting. The group of slowly migrating 4S RNAs yielded closely similar fingerprints characteristic of $tRNA^{Leu}$ (unpublished data). These $tRNA^{Leu}$ spots presumably differ slightly in their states of maturation or possess different conformations in these partially denaturing 4 M urea gels. The single, faster moving component yielded the RNase T1 oligonucleotides typical of $tRNA_1^{Met}$ of *X. laevis* (refs. 4 and 11; see Fig. 6).

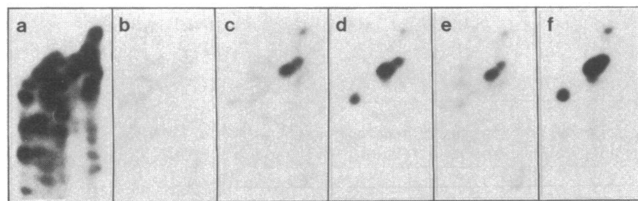

FIG. 3. Two-dimensional gel electrophoretic analysis of somatic 4S RNA and of 4S RNA obtained from oocytes injected with tDNA fragments. (*a*) Somatic tissue culture cells; (*b*) control oocytes. Oocytes injected with: (*c*) *Alu* I fragment A; (*d*) *Alu* I fragment A and *Hpa* II fragment A; (*e*) *Alu* I fragment A and fragment c of Fig. 1; (*f*) *Alu* I fragment A and fragment c linked to h22 sea urchin histone DNA.

Removal of 5′ Flanking Sequences Abolishes $tRNA_1^{Met}$ Gene Expression. Having established that the *Hpa* II fragment A, consisting of the structural gene for $tRNA_1^{Met}$ together with flanking sequences several hundred nucleotides long, is capable of producing faithful RNA transcripts, we attempted to narrow down the sequences that are essential for expression of the gene. Endonuclease *Hin*fI cleaves the *Hpa* II fragment A twice near the 5′ end of the structural gene, once near position −21 within the sequence 5′ G-A-A-T-C 3′, and also near position −120 within the sequence 5′ G-A-T-T-C 3′. Seventy-six base pairs downstream from the 3′ end of the structural gene, there is another *Hin*fI recognition site 5′ G-A-G-T-C 3′ (ref. 3, unpublished data; see Fig. 1).

Because the three *Hin*fI recognition sequences are all different, the restriction products do not rejoin in a random fashion upon ligation, but, with one exception, form DNA of the original conformation. Before ligation, the *Hpa* II fragment A was treated with bacterial alkaline phosphatase to prevent ligation via the *Hpa* II restriction site and then digested with *Hin*fI. Fragments a, b, c, and d (see Fig. 1 and Fig. 2, slot a) were isolated and ligated together in various combinations. Whereas fragment a can link with fragment b only in the correct orientation, inspection of the *Hin*fI sequences reveals that fragment b can recombine with fragment c in both orientations or with two fragments c at either end to yield the molecule c-b-c. Because we were anticipating an all-or-none response from the combination b-c in one orientation, the presence of these additional combinations was of no major concern and, indeed, proved to be unimportant.

Aliquots of the ligated molecules were precipitated together with an equimolar amount of *Alu* I fragment A to which, in some experiments, histone DNA had also been added as a carrier. The precipitated DNA was dissolved in injection buffer containing [α-^{32}P]GTP and the solution was injected into the nucleus of centrifuged oocytes. The DNA a-b-c injected into the oocyte nucleus produced $tRNA_1^{Met}$ at a rate to similar to that of the untreated *Hpa* II fragment A (Fig. 4). Hence, fragment d near the 3′ end of the *Hpa* II fragment is not essential for the expression of the $tRNA_1^{Met}$ gene. Next, the combination b-c was tested in which fragment c containing the structural gene was

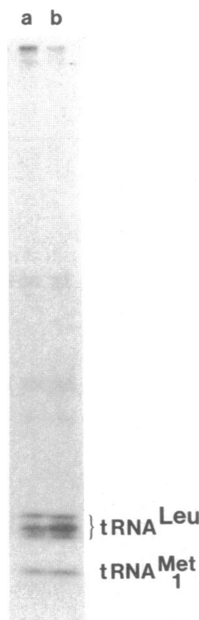

FIG. 4. One-dimensional gel analysis of 4–5S RNA obtained from injected oocytes. *Alu* I fragment A was injected together with *Hpa* II fragment A (lane a) or the ligated fragments a-b-c (lane b). Electrophoresis was through a 10% polyacrylamide/4 M urea gel.

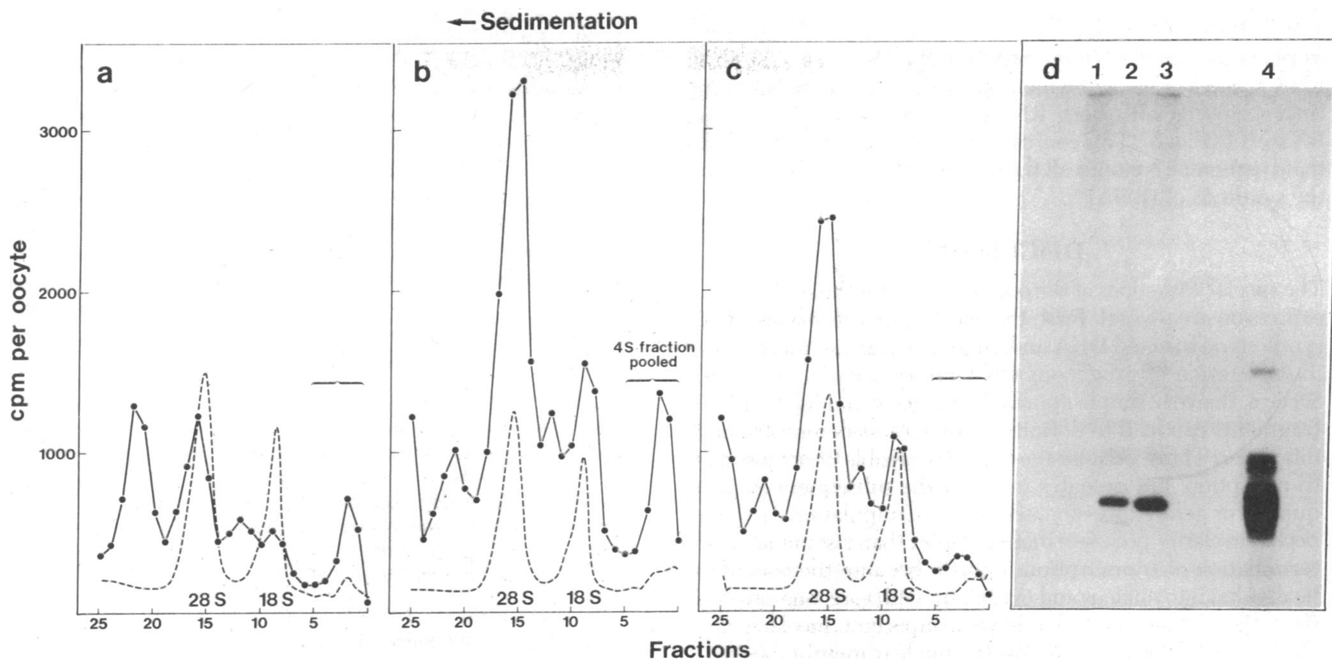

FIG. 5. Sucrose gradients of RNA obtained from injected oocytes and one-dimensional gel analyses of pooled 4S fractions. Oocytes were injected with pCR1 alone (*c* and *d*, lane 1); the recombinant pCR1-tmetA1, 3-hr incubation (*a* and *d*, lane 2); or pCR1-tmetA1, 16-hr incubation (*b* and *d*, lane 3). The 10% polyacrylamide/7 M urea gel shown in *d* included *X. laevis* somatic 4–5S RNA (lane 4).

extended at the 5′ end by the 99-base-pair fragment b. The results obtained with this combination were variable. At maximum activity, this DNA produced as much $tRNA_1^{Met}$, relative to $tRNA^{Leu}$ as did the *Hpa* II fragment A (Fig. 4). Because the ligation products b-c were active most of the time, we conclude that section a of the 5′ flanking sequence is not essential for the transcription of the $tRNA_1^{Met}$ gene. When fragment c, which contains only an additional 22 base pairs adjacent to the 5′ end of the structural gene, was injected into oocytes, no $tRNA_1^{Met}$ could be detected.

We considered, first, that the inactivity of fragment c could be caused by our removal of a sequence vital to the initiation of $tRNA_1^{Met}$ transcription. If true, this would place the sequence promoting transcription within fragment b, possibly at the interface between b and c. Second, we thought it possible that the essential sequences were still present in fragment c, but could not act for one of two reasons. (*i*) Linear molecules injected into the oocyte nucleus are degraded by exonucleases (12). The survival time of short tDNA fragments has not been determined. In principle, therefore, degradation of the tDNA could account for the variable success of the experiments in which fragments b-c were injected. Furthermore, if promoter sequences were present at the 5′ terminus of fragment c, they would be rapidly destroyed. (*ii*) Conceivably, the inactivity of fragment c could be due to failure of RNA polymerase III to interact with the recognition sequences placed at the end of a short DNA molecule.

Fragment c Contains All Essential Information for Synthesis of $tRNA_1^{Met}$. To eliminate these two possible effects, we extended the 5′ end of fragment c by ligating it to a DNA molecule with an appropriate *Hin*fI site. Such a DNA fragment containing the right-hand portion of the h22 histone clone (13) was linked to fragment c in opposite polarity. Injection of this fragment c extended by histone DNA, together with *Alu* I fragment A as a control, yielded two prominent spots on a two-dimensional gel, characteristic for $tRNA^{Leu}$ and $tRNA_1^{Met}$ (Fig. 3, slot f). The assignment of $tRNA_1^{Met}$ was confirmed by RNase T1 fingerprinting (results not shown). Thus, the DNA sequences contained within fragment c are capable of directing the synthesis of $tRNA_1^{Met}$.

We established previously that cloned histone DNA of the sea urchin, when injected into the oocyte nucleus of *X. laevis*, is transcribed exclusively by RNA polymerase II, while injected cloned tDNA is transcribed by RNA polymerase III (14). It might be argued that ligation of gene fragment c to the histone DNA created a spurious promoter sequence for polymerase III or that a promoter sequence for polymerase III preexisted in the histone DNA which was then revealed by addition of the structural gene for tRNA synthesis. In order to refute these unlikely possibilities, we placed gene fragment c into a different genetic background by integration into the bacterial plasmid pCR1. First, the staggered *Hin*fI ends of the fragment were filled in by means of DNA polymerase (5, 15) and the *Eco*RI linkers G-G-A-A-T-T-C-C were added by flush-end ligation (ref. 16; see *Materials and Methods*). The newly created *Eco*RI sticky ends were then used to introduce fragment c into the *Eco*RI site of pCR1. The recombinant plasmid DNA, named pCR1-tmetA1, was prepared and assayed for $tRNA_1^{Met}$ production in injected oocytes. Injection of plasmid pCR1 alone resulted in little labeled 4S RNA, whereas oocytes injected with the recombinant DNA produced 4S RNA at a high rate during

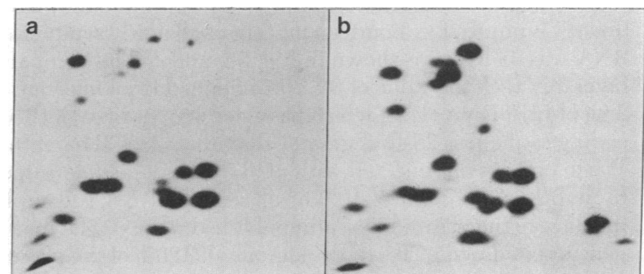

FIG. 6. RNase T1 oligonucleotide fingerprint analyses of $tRNA_1^{Met}$. (*a*) [α-^{32}P]GTP-labeled $tRNA_1^{Met}$ obtained from oocytes injected with pCR1-tmetA1 (see Fig. 5). (*b*) Uniformly ^{32}P-labeled $tRNA_1^{Met}$ from *X. laevis* tissue culture cells, purified by hybridization to the cloned t210 tDNA (2, 4). The 3′-terminal oligonucleotide does not contain a G residue and is therefore not labeled in fingerprint *a*. Minor differences between the two fingerprint patterns are presumably due to incomplete modification of some oligonucleotides in the injected sample.

3-hr or 16-hr incubations (Fig. 5). When analyzed by gel electrophoresis, this RNA migrated predominantly as a single 4S RNA species (Fig. 5*d*) which was identified as $tRNA_1^{Met}$ by fingerprinting with both RNase A (results not shown) and RNase T1 (Fig. 6). This confirms the conclusion reached above that fragment c contains all the genetic information needed for the synthesis of $tRNA_1^{Met}$.

DISCUSSION

The special attractions of the oocyte system for the study of gene expression are several. First, the oocyte supports massive RNA synthesis on injected DNA and, in some instances, there is good interpretation of exogenous DNA information (1, 4, 17–20). Second, the very simple approach of cutting and ligating DNA fragments can lead to definitive answers, as demonstrated in this paper. Third, because the oocyte is capable of not just mere transcription, but executes many of the subsequent steps required for gene expression, sequence manipulation can be expected to clarify processes more complex than just initiation and termination of transcription. Fourth, because the oocyte can be divided into nucleus and cytoplasm with ease, one can study the rather subtle, but probably very important, mechanisms of selective RNA transport across the nuclear membrane (14).

Cloned tDNA, when injected into the oocyte, is transcribed exclusively by RNA polymerase III (14). tRNAs produced from injected templates are of correct size, are at least partially methylated, and accumulate rapidly in the oocyte (4, 19, 20). In bacteria, RNAse P processes the 5′ end of tRNA precursors probably by recognizing the structure of the tRNA moiety within the precursors rather than by identifying the nucleotide composition of the excess sequences (21). There exists the formal possibility that correctly processed tRNA is produced in the oocyte through the editing of random transcripts, rendering the search for regulatory sequences illusory. However, when heterologous yeast tDNA is injected into oocytes, the precursor tRNA has a unique 5′-tetraphosphate terminus (20), showing that tDNA transcription is initiated in a very precise manner within the oocyte.

In this paper we have narrowed down the minimum sequence required for the faithful production for $tRNA_1^{Met}$ to a relatively short DNA segment. Interestingly, DNA fragment c, when ligated to histone DNA, produced amounts of $tRNA_1^{Met}$ similar to those produced by *Hpa* II fragment A, which contains the natural flanking sequences (compare Fig. 3 *d* and *f*). Hence, fragment c can behave autonomously for the synthesis of $tRNA_1^{Met}$. Moreover, there is no evidence that deletion of the sequences further upstream grossly impairs the rate of $tDNA_1^{Met}$ transcription.

In absolute terms, transcription of the recombinant pCR1-tmetA1 is impressive. Fourteen percent of all newly synthesized RNA was 4S RNA, as shown in Fig. 5 *b* and *c*. This compares favorably with the value of 30–70% obtained from injection of 2 ng of t210 DNA (1, 4), which has a density per mass of tRNA coding sequences 28-fold greater than that of pCR1-tmetA1. When oocytes are injected with pCR1, it may be determined by hybridization that 5–10% of all cellular RNA is complementary to the plasmid DNA (unpublished results). The tmetA1 gene inserted into pCR1 represents only 1/120th of the plasmid DNA mass, and yet it produces twice as much RNA as does the bacterial DNA.

In view of the rather complex nature of prokaryotic promoters (22), it is surprising that a eukaryotic gene fragment containing only 22 bases in excess of the structural gene is still capable of faithful expression of its genetic information. Possibly, transcription of the $tDNA_1^{Met}$ gene is constitutive and does not, in fact, require a complicated regulatory mechanism. We may also have to consider the possibility that the recognition between DNA and RNA polymerase may not depend on the 5′ sequences outside the structural gene, as the classical view demands, but may be determined by the structural gene itself. Because the $tDNA_1^{Met}$ contains additional, topologically interesting restriction sites that invite further manipulation, the second hypothesis can be put to test.

We thank Dr. Margaret Chipchase for critical reading and Mrs. S. Oberholzer for expert preparation of the manuscript. We are grateful to Mr. F. Ochsenbein for his photography and skillful drawings. We thank Mrs. Elisabetta Di Capua for preparing the recombinant DNA. R.A.K. is a Fellow in Cancer Research supported by Grant DRG-196-F of the Damon Runyon-Walter Winchell Cancer Fund. This work was supported by a grant of the State of Zürich and by the Swiss National Research Foundation, Grant 3.257.077.

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