Transcription of class III genes in cell-free extracts from the nematode *Caenorhabditis elegans*

Barry M.Honda, Robert H.Devlin, Donald W.Nelson and Meenal Khosla

Department of Biological Sciences, Simon Fraser University, Burnaby, British Columbia V5A 1S6, Canada

Received 16 August 1985; Revised and Accepted 6 December 1985

ABSTRACT

Using the nematode *Caenorhabditis elegans* as a model organism, we have prepared cell-free extracts which accurately transcribe cloned homologous 5S RNA genes in vitro. These extracts also transcribe cloned tRNA genes, and actively process the resulting products. Unlike tRNA genes, transcription of 5S DNA shows some species specificity: *C. elegans* extracts do not transcribe *Xenopus* 5S RNA genes, nor does a *Xenopus* extract efficiently transcribe the heterologous nematode 5S DNA. However, addition of limiting amounts of *C. elegans* fractions permits *Xenopus* extracts to transcribe nematode 5S RNA genes. This apparent biochemical "complementation" may provide an assay to purify 5S RNA gene-specific factors from *C. elegans*.

INTRODUCTION

As a first step toward understanding transcription and its regulation in *C. elegans*, we have prepared and characterized cell-free nematode extracts which transcribe 5S RNA and tRNA genes in vitro.

We have chosen to work with a nematode expression system because *C. elegans* has many advantages as a model organism, amenable both to biochemical and to extensive genetic analyses (1,2). Recent advances--determination of the complete embryonic cell lineage (3), work on a restriction map of the entire genome, and promising results with gonadal transformation by cloned genes--provide further powerful tools to study gene expression. Furthermore, we believe that it is important to work with a homologous system, even though it is possible to study the expression of *C. elegans* genes in available heterologous expression systems. While the internal promoter regions in nematode tRNA genes have been elegantly described using heterologous *Xenopus* expression systems (e.g. ref 4),

there can be important species-specific effects of flanking DNA, which require homologous systems to detect and study. Examples include mammalian rRNA genes transcribed by RNA polymerase I (5), *Drosophila* heat shock genes read by RNA polymerase II (6), and tRNA genes in *Bombyx mori* (7) and *Drosophila* (8).

Work with cell-free extracts has provided many important insights into the DNA sequences and protein factors required for transcription (reviewed in 9). In the case of RNA polymerase III (pol III), 5S RNA and tRNA genes possess internal promoter regions which interact with multiple soluble transcription factors(9). In *Xenopus*, a specific factor TFIIIA interacts with the 5S RNA gene (10); detailed studies on the mechanism of factor-DNA interactions indicate the formation of different stable and rate-limiting complexes. Such complexes may explain the developmental switching of 5S RNA gene types in *Xenopus* (11, 12). The recent cloning of the gene for TFIIIA makes possible further studies of its expression and regulation in development (13).

We noted in early experiments that *C. elegans* 5S RNA genes were not well transcribed by *Xenopus* extracts. The successful characterization of RNA polymerases from *C. elegans* (14) led us to try to establish cell-free transcription extracts from this animal. Our extracts do transcribe *C. elegans* 5S RNA genes; they also transcribe and process tRNAs, and can be used to screen cloned, uncharacterized *C. elegans* DNAs for putative pol III promoters.

We were initially surprised to find that extracts from *Xenopus* or *C. elegans* cannot transcribe each others' (i.e. heterologous) 5S DNAs efficiently. There is apparently no such problem with tRNA genes (4, and our results). Moreover, the addition of limiting amounts of *C. elegans* extract to *Xenopus* extracts biochemically complements these extracts and allows transcription of the heterologous nematode 5S RNA gene. If a nematode-specific 5S RNA gene factor is involved, this result may allow us to purify such a protein from *C. elegans*.

MATERIALS AND METHODS

Preparation of Nematode Extracts. *C. elegans* var. (Bristol) N2, or mutant strain ncl-1(e1865) were grown on high-peptone plates

(15). Eggs were prepared from harvested adults by hypochlorite treatment (16), frozen in liquid nitrogen, and stored at -80 C.

Eggs were quick thawed and passed twice at 10,000 psi through a French Press cell in 9 volumes of homogenization buffer: 50mM Tris pH 7.9, 0.1mM EDTA, 25% glycerol, 10mM $MgCl_2$, 0.4M ammonium sulfate, 1 mM phenylmethylsulfonyl-fluoride (PMSF), and 5mM dithiothreitol (DTT). After centrifugation at 100,000xg for 90min, solid ammonium sulfate (0.35g/ml) was slowly added to the supernatant. The resulting precipitate was redissolved in the original egg volume of Buffer PC (40mM Tris pH 7.9, 1mM EDTA, 20% glycerol) and dialysed against Buffer PC until the conductivity of the dialysate corresponded to Buffer PC-0.1M NaCl. The resulting extract (protein concentration approx. 30-40 mg/ml) was aliquoted and stored at -80 C.

Transcription Assays. Assays using *Xenopus* extracts were performed as described by Engelke *et al* (10). Unless otherwise specified, nematode assays contained in 50 μ l: 5-10 μ l of extract, 50mM Tris pH 7.9, 3 mM $MgCl_2$, 0.5mM DTT, 0.6mM each of ATP, CTP and UTP, 15 μ M GTP, 5-10 μ Ci of alpha- ^{32}P -GTP, 0.2 μ g/ml of alpha-amanitin, and 0.5mM spermine. Plasmid DNAs were usually added to a concentration of 25 μ g/ml. Incubations were at 25 C for 45-60min, and were stopped by adding sodium dodecyl sulfate (SDS) to 1%. To follow nucleolytic processing of precursor RNAs, after synthesis a chase of cold GTP was added (final conc. = 2mM), and reactions were incubated further at 25 C for times indicated.

Gel Electrophoresis. Samples were extracted with an equal volume of chloroform-phenol (1:1), mixed with marker dyes, and loaded directly onto polyacrylamide gels (either 8, 10 or 12%) in Tris-borate (TBE) buffer pH 8.3 (90mM Tris-borate, 0.1mM EDTA), including 0.1% SDS. In some cases (e.g. when sizing RNA products), samples were ethanol precipitated and loaded under denaturing conditions onto TBE gels containing 7M urea (5).

Except for slightly sharper bands, results were essentially the same.

Fingerprinting of RNA Transcripts. For analysis of *in vitro* products, scaled-up transcription reactions (500 μ l) were

electrophoresed on preparative polyacrylamide gels, and after brief exposure to X-ray film, the radioactive band(s) was excised and RNA eluted by incubation in 1% SDS, 1M NaCl (17). The resulting RNA was then precipitated, digested with RNase T1, and analysed by electrophoresis on cellulose acetate strips followed by homochromatography on DEAE cellulose plates (Machery-Nagel) (18). In vivo 5S RNA samples were labelled by feeding ^{32}P -phosphate labelled E. coli C600 to nematodes, followed by RNA isolation and analysis as above (17).

Plasmid DNA Templates. pXbs1 contains one cloned Xenopus borealis somatic 5S DNA repeat (19) in the vector pBR322(20). pXltmet has a single Xenopus laevis tRNAm^{et} gene (10). pCe5S has a single C. elegans 5S DNA repeat and pCe5ST a portion of the C. elegans 5S DNA repeat. The latter contains coding and 5' flanking DNA, but is missing a 3' run of T's (21), a putative termination signal for pol III. pMK8 and pMK14 are two of several subclones into pUC13 (22) which potentially contained C. elegans tRNA genes (R. Devlin and M. Khosla, unpublished results).

RESULTS

Xenopus Extracts Transcribe C. elegans 5S DNA Inefficiently.

After isolating 5S RNA genes from C. elegans (21), we first tried to transcribe them in extracts prepared from whole Xenopus oocytes. The results are shown in Fig. 1. Whereas the Xenopus gene in pXbs1 is actively transcribed, upon normal film exposures there is no evidence of transcription of pCe5S or pCe5ST. After prolonged overexposure (compare with the pXbs1 lane), a faint signal is observed. The expected product from pCe5S is 120nt long. If transcription were to start in the correct place in pCe5ST which lacks a 3' terminator, there are two sequences further downstream in the vector about 185 and 225bp away which have runs of T residues. The sizes of products from pCe5S and pCe5ST (approx. 120 and 185nt respectively) were as expected. We conclude that Xenopus extracts can accurately initiate and transcribe C. elegans 5S RNA genes, but very inefficiently. Drosophila cell extracts were similarly unable to efficiently transcribe pCe5S (results not shown).

Fig.1. Transcription of cloned 5S RNA genes in *Xenopus* oocyte extracts. The indicated template was incubated in oocyte extracts, and radioactive transcription products were separated on polyacrylamide gels as indicated in Materials and Methods. The resulting autoradiograph was exposed for 4 days on Kodak XAR-5 film with an intensifying screen (23).

Homologous Cell-free Extracts Transcribe *C. elegans* 5S RNA Genes

Attempts to prepare active extracts from mixed populations (eggs, larvae and adults) of nematodes were unsuccessful, even after fractionation trials to remove nucleases and putative inhibitors. These attempts were made both with wild-type N2 animals, and with a *ncl-1(e1865)* mutant strain with abnormal (enlarged) nucleolar morphology (generously provided by Dr. Ed Hedgcock). The latter strain was initially chosen for extract

Fig. 2. Template-dependent transcription of cloned *C. elegans* 5S RNA genes in homologous extracts.

The indicated templates--(-)=no DNA, pB=pBR322, 5S=pCe5S and 5ST=pCe5ST --were incubated in nematode extracts and radioactive transcripts analyzed as above. The resulting autoradiograph was exposed for 16 hr. as in Fig. 1.

Fig. 3. T1 fingerprint analysis of in vivo versus in vitro synthesized 5S RNA. ^{32}P -labelled RNA prepared as in Materials and Methods was digested with RNase T1 and the resulting oligonucleotides were fingerprinted as described (18). In vivo samples were labelled by feeding nematodes with bacteria grown in inorganic ^{32}P -phosphate (upper panel), while in vitro samples were labelled with $\alpha\text{-}^{32}\text{P}$ -GTP (lower panel). The arrow in the upper panel indicates the extra spot representing the 3' CU-OH in the in vivo sample. Cellulose acetate electrophoresis originated from the bottom left corner, and proceeded from left to right. After transfer, homochromatography was performed upwards, along the vertical axis.

preparations because of a potential RNase deficiency.

Extracts prepared from frozen eggs (either ncl-1 or N2) were, however, active in transcribing cloned C. elegans 5S RNA genes. Typically, from 200 high-peptone plates, we obtained 1.5 to 2ml of eggs and hence about 2 to 3ml of extract. Fig. 2 shows that these extracts support the DNA-dependent synthesis of a 5S RNA-sized product from pCe5S. Note the lack of signal in the minus DNA or pBR322 lanes. Moreover, the predicted products of approx. 185nt and 225nt can be seen from pCe5ST. We took this as evidence that the nematode extract was initiating 5S RNA synthesis at or near the correct site. The efficiency of transcription was not influenced by the state of the template; supercoiled or nicked circles and linear DNA were all comparable (results not shown).

To further confirm that accurate transcription of 5S RNA genes occurs, the fingerprints of in vivo and in vitro synthesized 5S RNA were compared. They show a clear correspondence (Fig 3). The only major difference is an extra spot in the in vivo sample, which represents the 3' CU-OH (not labelled in the in vitro reactions, which contained ^{32}P -GTP). This further suggests that 5S RNA is correctly synthesized in these extracts.

Fig. 4 shows a typical time course for synthesis of 5S RNA in the cell-free system. The best temperature for synthesis is 25 C, with the reaction levelling off at about 90min. Optimal concentrations of KCl (60mM), magnesium (3mM) and spermine (0.5mM), and template DNA (25-50ug/ml) were also determined. These optima are not very different from those described for *Xenopus* or human systems (9).

Nematode Extracts Transcribe and Process tRNAs. Fig. 5 shows that C. elegans extracts will also transcribe cloned tRNA genes, and process the resulting products. In Fig. 5, the first four lanes show results with a *Xenopus* extract for the synthesis and processing of tRNA^{met} from a cloned *Xenopus* tRNA^{met} gene (pXltmet). After 45min of synthesis, a chase of cold GTP (2mM) was added, and samples were incubated further to monitor processing. With a chase of cold GTP, label moves from upper precursor band to tRNA product.

Fig. 4. Time course of transcription in *C. elegans* extracts. pCe5S was transcribed in nematode extracts for the indicated times, and ^{32}P transcription products separated on polyacrylamide gels. After autoradiography, radioactive 5S RNA bands were cut out and Cerenkov counted in a scintillation counter.

Fig 5. Transcription and processing of tRNAs in *C. elegans* extracts. (a) The indicated templates (tmet=pXlmet, MK8 or MK14=putative cloned *C. elegans* tRNA^{met} genes) were transcribed in either *Xenopus* extracts (lefthand 4 lanes) or *C. elegans* extracts. MK14 was incubated for 45 min, followed by addition of a chase of cold GTP (to 2mM) to monitor processing. For MK8, no cold chase was used. At the times (in min) indicated above the lanes, samples were processed as described in Figure 2.

Fig. 6. Evidence of species specificity in 5S RNA gene transcription. The indicated templates (Ce5S=pCe5S, XBS=pXbs1, tmet=pXltmet) were transcribed in either *C. elegans* (left) or *Xenopus* oocyte (right) extracts and products analyzed as described in Fig. 2.

The next three lanes show synthesis and processing of the same gene, but by a nematode extract, which gives the same products. The last three lanes show that the nematode extracts also work with a putative cloned nematode tRNA gene (MK14). In this case *C. elegans* extracts were used to screen plasmid subclones from recombinant phage thought to contain tRNA genes. Of several subclones tested, two of independent origin, MK8 and MK14, acted as excellent templates, while other plasmids were inactive. On the right of Fig. 5, no chase of cold GTP was provided during synthesis from MK8. We did not observe any lag before transcription started. Note also that processing continues after synthesis has levelled off. Addition of further nucleotide triphosphates led to more synthesis, suggesting that nucleotides become limiting in the reaction (results not shown). However, it is also possible that the processing activities are relatively more stable than transcriptional components in the extract.

Species-specific Effects in 5SRNA Transcription in vitro. We observed initially that *Xenopus* extracts would not transcribe 5S

<u>X. laevis</u>	GATCTCGGAAGCCAAGCAGGGTCGGGCTGGTTAGTACTTGGATGGGAGACCGCCTGGGA
	* * ** ** * * * *
<u>C. elegans</u>	GATCTGGCAAGTTAAGCAACGTTGAGTCCAGTTAGTACTTGGATCGGAGACGGCCTGGGA
	**** * * * * * *
<u>D. melanogaster</u>	GATCACCGAATTAAGCAGCGTCGGGCGCGGTTAGTACTTAGATGGGGGACCGCTTGGGA
	50 60 70 80 90 100

Fig. 7. Species variation between internal sequences of 5S RNA genes. Sequence data for 5S RNA genes of Xenopus, pXBS1 (19), C. elegans (21), and Drosophila (24). The black line indicates areas protected by TFIIIA binding (5), while asterisks mark nucleotide differences between pairs.

RNA genes from C. elegans, while transcription of C. elegans tRNA in a Xenopus system is well documented (9). Fig. 6 shows that while tRNA^{met} is synthesized in both Xenopus and nematode extracts, the respective heterologous 5S RNA genes are not. The internal promoter sequences of Xenopus and C. elegans 5S DNA differ considerably (Fig 7); this may explain the relative "incompatibility" of the two systems. A number of differences also occur between nematode and Drosophila 5S DNA (Fig. 7),

Fig. 8. Transcription of 5S RNA genes by Xenopus extracts +/- added C. elegans extracts. In each lane, template was transcribed, and radioactive transcription products isolated and analyzed as described in Fig. 2. In lanes 1 and 2 a Xenopus oocyte extract (20ul) was used: lane 1 with pXbs1, lane 2 with pCe5S. In lane 3, as a control, 1ul of C. elegans extract alone was used with pCe5S DNA. In lane 4, 1ul of C. elegans extract was added to 20ul of Xenopus oocyte extract with pCe5S as template.

which may account for the poor synthesis of nematode 5S RNA by fly extracts.

Is the problem a lack of factors which can recognize and interact specifically with the internal promoter region? In Fig. 8, addition of small amounts of C. elegans extract to *Xenopus* extracts allowed transcription of the heterologous pCe5S. This biochemical "complementation" suggests the possibility of interactions between a nematode-specific 5S DNA factor and its homologous gene.

DISCUSSION

We have chosen to study transcriptional regulation in C. elegans as an important model organism, readily amenable to biochemical, genetic and developmental studies. We have prepared and characterized homologous cell-free systems for the class III (5S and tRNA) genes as a first step toward this goal. Optimal assay conditions and RNA synthetic activity are not significantly different from those described for *Xenopus* and human systems (9).

C. elegans and *Xenopus* extracts were unable to transcribe 5S RNA genes from heterologous sources (*Xenopus* and C. elegans, respectively). However, the addition of C. elegans extract complemented *Xenopus* extracts and permitted pCe5S transcription. While other explanations of this result are possible (other factors involved etc.), we take this as preliminary evidence of a species-specific 5S DNA factor. Such a result may also provide an assay to purify such a protein from C. elegans. The assay, complementing an intact *Xenopus* extract with "helper" nematode fractions to transcribe pCe5S, is analogous to that first used to purify *Xenopus* TFIIIA (10). This avoids problems with fractionation of a single extract where instability of multiple components can lead to loss of transcription activity. We are also screening C. elegans libraries with a *Xenopus* TFIIIA cDNA clone (13), in attempts to isolate and study the corresponding nematode gene.

Attempts to show a similar complementation effect, by adding *Xenopus* TFIIIA to a nematode extract, failed to give any transcription of pXbS1, the *Xenopus* 5S RNA gene (results not

shown). This may reflect poor recognition of TFIIIA by the nematode components of the extracts, or failure on our part to properly optimize transcription.

The availability of homologous nematode extracts allows experiments to define important DNA sequences and other interacting transcription factors in C. elegans. It also provides a system to study the synthesis and processing of precursor tRNAs, and a potentially rapid assay for pol III promoters in cloned, uncharacterized C. elegans DNAs. Finally, it provides a starting point from which to develop systems in C. elegans to study the transcription of genes by RNA polymerase II. In combination with other experimental tools available with this organism (as outlined in the Introduction), such work should further our understanding of gene regulation in eukaryotes.

ACKNOWLEDGMENTS

We wish to thank Drs. Shizu Hayashi and G. Tener for assistance with RNA fingerprinting, G. Spiegelman for help with Drosophila extract transcriptions, Ed Hedgcock for the strain ncl-1, and D.L. Baillie, J. Segall, R.G. Roeder and colleagues for helpful discussions. This work was supported by grants to BMH from NSERC Canada, and a Simon Fraser University President's Research Grant.

REFERENCES

1. Brenner, S. (1974) Genetics 77, 71-94.
2. Marx, J.L. (1984) Science 225, 40-42.
3. Sulston, J.E., Schierenberg, E., White, J.G., Thomson, J.N. (1983) Dev. Biol. 100, 64-119.
4. Ciliberto, G., Castagnoli, L, Melton, D.A. and Cortese, R. (1982) Proc. Natl. Acad. Sci. USA 79, 1195-1199.
5. Mishima, Y, Financsek, I., Kominami, R. and Muramatsu, M. (1982) Nucl. Acids Res. 10, 6659-6670.
6. Dudler, R. and Travers, A.A. (1984) Cell 38, 391-398.
7. Sprague, K.U., Larson, D. and Morton D. (1980) Cell 22, 171-178.
8. Schaack, J., Sharp, S., Dingermann, T., Burke, D.J., Csoley, L. and Soll, D. (1984) J. Biol. Chem. 259, 1461-1467.
9. Heintz N. and Roeder, R.G. (1982) in Genetic Engineering, Setlow and Hollaender, eds, Vol. 4 pp 57-89, Academic Press, New York.
10. Engelke, D.R., Ng, S., Shastry, B.S. and Roeder, R.G. (1980) Cell 19, 717-728.

11. Brown, D.D. (1984) Cell 37, 359-365.
12. Bieker, J.J., Martin, P.L. and Roeder, R.G. (1985) Cell 40, 119-127.
13. Ginsberg, A.M., King, B.O., and Roeder, R.G. (1984) Cell 39, 479-489.
14. Sanford, T., Golomb, M. and Riddle, D.L. (1983) J. Biol. Chem. 258, 12804-12809.
15. Rose, A.M., Baillie, D.L., Candido, E.P.M., Beckenbach, K.A. and Nelson, D.W. (1982) Mol. Gen. Genet. 188, 286-291.
16. Swanson, M.M. and Riddle, D.L. (1981) Dev. Biol. 84, 27-40.
17. Sulston, J.E. and Brenner, S. (1974) Genetics 77, 95-104.
18. Brownlee, G.G. (1972) in Laboratory Techniques in Biochemistry and Molecular Biology, T.S. Work and E. Work eds., Vol. 3, pp. 1-265, Amsterdam: North Holland.
19. Doering, J. (1977) Carnegie Inst. Wash. Yearbook 76, 120-125.
20. Bolivar, F., Rodriguez, R.L., Green, P.J., Betlach, M.C., Heynecker, H.L., Boyer, H.W., Crosa, J.H. and Falkow, S. (1977) Gene 2, 95-113.
21. Nelson, D.W. and Honda, B.M. (1985) Gene, in press.
22. Viera, J. and Messing, J. (1982) Gene 19, 259-268.
23. Laskey, R.A. and Mills, A.D. (1977) FEBS Lett. 82, 314-316.
24. Tschudi, C. and Pirrotta, V. (1980) Nucl. Acids Res. 8, 441-451.