Transcription of genomic bovine and *Xenopus laevis* DNA species by RNA polymerase III in HeLa-cell cytosol extracts

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RNA transcribed from genomic *Xenopus laevis* DNA by RNA polymerase III in HeLa-cell extract was found in discrete size classes and was transcribed from at least two different *Xenopus* repeat DNA species. Very little 5S ribosomal RNA was transcribed, contrasting with results obtained on transcription of genomic *Xenopus* DNA by *Xenopus* extract [Bogenhagen *et al.* (1982) Cell **28**, 413–421]. The low transcription was not due to an inability to use 5S rDNA templates, since the cloned *Xenopus* 5S ribosomal gene and pseudogene were effective templates for RNA polymerase III in HeLa extract. RNA transcribed from genomic bovine DNA by RNA polymerase III in HeLa-cell cytosol extract consisted of 120-nucleotide RNA and a larger amount of heterogeneously sized RNA (180–650 nucleotides). Only a small portion of the 120-nucleotide RNA was 5S rRNA. Most of the 120-nucleotide RNA and the larger RNA species were transcribed from one bovine repeat DNA. Genes for 5S rRNA and bovine repeat DNA were transcribed roughly in proportion to their frequency in *Bos*, contrasting with results in a homologous system in which transcription of repeat genes is repressed [Furth (1985) Biochem. Biophys. Res. Commun. **131**, 551–556]. Bovine 5S rRNA genes appear to be concentrated on one DNA fragment obtained by restriction-enzyme-*Hind*III digestion of genomic bovine DNA.

INTRODUCTION

RNA polymerase III, one of three eukaryotic RNA polymerases, is responsible for the synthesis *in vivo* of 5S rRNA, tRNA, 7S RNA (4.5 S RNA in rodents) and a number of small RNA species transcribed after viral infection (Zieve, 1981; Ciliberto *et al.*, 1983). In addition to these RNA species, RNA polymerase III transcribes a class of repetitive DNA species, the prototype of which is the human Alu repeat (Jelinek & Schmid, 1982; Singer, 1982), although the vast majority of this class of repetitive DNA appears to be silent, for most of the time, in most cell types.

Synthesis of these RNA species can be reproduced *in vitro* by using either cytosol or whole cell extracts. Purified RNA polymerase and DNA template are insufficient, since purified RNA polymerase III does not correctly initiate transcription unless other components, found in cytosol or whole cell extracts, are present (Wu, 1978; Weil *et al.*, 1979).

Most of the studies *in vitro* from which these conclusions were drawn were carried out with cloned genes. Only a limited number of genes are transcribed by this polymerase, and in cloning these genes, 5' or 3' sequences required for, or that may inhibit, transcription *in vivo* may have been lost. Initial results on transcription of genomic bovine DNA by RNA polymerase III in HeLa-cell cytosol extract indicated that, in addition to large heterogeneous RNA (of about 180–650 n), significant amounts of 5S-sized RNA (120 n) were synthesized (Furth & Su, 1983; Furth, 1985). Further studies, reported here, indicate that most of the 120 n RNA transcribed from genomic bovine DNA was transcribed

from one widely dispersed bovine repeat, although a small amount of 5S rRNA was transcribed. Surprisingly little 5S rRNA was transcribed from genomic *Xenopus* DNA, contrasting with results obtained on transcription by *Xenopus* oocyte nuclear extract (Bogenhagen *et al.*, 1982). Instead, transcription was predominantly of at least two, possibly more, classes of repeat DNA.

MATERIALS AND METHODS

Calf thymus was obtained from a local abattoir. Fetal-calf thymus, calf liver and bovine lymphosarcoma were obtained through the courtesy of Dr. J. Ferrer of the Veterinary School of this Institution. Glioblastoma (T98G) (Stein, 1979) cells were obtained through the courtesy of Dr. L. Bello, also of the Veterinary School.

Genomic DNA was isolated by methods described previously or as described by Maniatis *et al.* (1982). *Xenopus laevis* erythrocyte DNA was prepared from nuclei obtained as described by Bogenhagen *et al.* (1982) and from tissue as described previously (Furth & Su, 1983). 5S rRNA was prepared from rat liver as previously described (Ackerman *et al.*, 1979) and from calf thymus by the same procedure, slightly modified.

pHU1075, a plasmid containing three head-to-tail copies of the sequenced *Xenopus laevis* oocyte 5S rDNA repeat unit x10 36, was a gift of D. Carroll, University of Utah. A plasmid containing a hamster 5S rRNA gene, pTH1 (Hart & Folk, 1982), was a gift of W. R. Folk. Plasmids containing bovine repeat DNA (pPGA7 and pPGB9) were gifts of J. E. Mayfield. pPGA7 contains 1.48 kb of bovine DNA, whereas the insert in pPGB9 is 3.4 kb (Richardson *et al.*, 1986). Plasmids were grown

Abbreviations used: n, nucleotide(s); kb, kilobase(s); bp, base-pair(s); SSC, 0.15 M-NaCl containing 15 mM-sodium citrate; TBE, buffer containing 89 mM-Tris-borate, 89 mM-boric acid and 2 mM-EDTA.

and DNA isolated by CsCl density-gradient centrifugation, essentially as described by Maniatis *et al.* (1982). A bovine DNA library, in Charon 28, grown in *Escherichia coli* K802, was a gift of F. Rottman, Case Western Reserve, Cleveland, OH, U.S.A. Cytosol extracts were obtained from HeLa cells grown in monolayers (Wu, 1978; Weil *et al.*, 1979; Furth & Su, 1983).

HeLa-cell cytosol extract efficiently transcribed plasmid DNA species containing a hamster 5S rRNA gene, a human tRNA gene and mouse Alu-type repeat DNA (results not shown). Human Alu repeat DNA (BLUR 8) was also transcribed, but less efficiently (Furth, 1985).

Routinely, RNA was synthesized in 25 or 50 μ l reaction volumes containing 50 mM-Tris/HCl, pH 7.9, 0.8 mM-EDTA, 2 mM-dithiothreitol, 30–40 mM-KCl, 4 mM-MgCl₂, the three unlabelled nucleoside triphosphates at 640 μ M, 40 μ M-[³²P]GTP or 20 μ M-[³²]UTP, 5 μ l of extract at 10 μ g of protein/ μ l (in a 25 μ l reaction volume) and DNA. Routinely 1 μ g of genomic DNA or 0.2 μ g of plasmid DNA per 25 μ l were used. After incubation (25 μ l reaction volume) for 60 min at 30 °C, 12.5 μ l of a 500 μ g/ml solution of proteinase K, 2.5 μ l of 1% SDS (to 0.04%) and 20 μ l of 5 M-urea were added and the mixture incubated for 15 min at 37 °C. A 1 μ l portion of a 10 mg/ml solution of yeast tRNA and 55 μ l of 1 M-ammonium acetate were then added, and the RNA was extracted with 2 vol. of phenol/chloroform (1:1, v/v); 2 vol. of 100% ethanol were added to the aqueous phase and the mixture was placed at –20 °C overnight. The RNA was then collected by centrifugation, resuspended in 0.5 ml of 70% (v/v) ethanol containing 10 mM-MgCl₂, mixed, and the suspension placed at –70 °C for at least 2 h. RNA was again collected by centrifugation, dried, resuspended in 90% (w/v) formamide containing glycerol, 1% (w/v) sucrose, 10 mM-EDTA, 0.1% Bromphenol Blue and 0.1% Xylene Cyanol, heated at 55 °C for 5 min and analysed on 10%-(w/v)-polyacrylamide gels in TBE buffer containing 0.26% bisacrylamide and 48% (w/v) urea (Maniatis *et al.*, 1982; Sanger & Coulson, 1978). The gels were radioautographed wet (usually for 16 h) at –70 °C by using Kodak X-Omat RP film with Lightning Plus intensifier screens. Unlabelled RNA species (tRNA and 5S rRNA) were located by u.v. absorption with the gel placed on an intensifier screen.

RNA was eluted from gels by either salt extraction or electro-elution. Salt extraction was carried out by placing the gel slice in 0.2 ml of 0.5 M-ammonium acetate containing 0.1% SDS and 0.1 mM-EDTA. After gentle agitation for 16 h at room temperature, the eluate was removed and elution repeated for 6–16 h. The eluates were combined and RNA was precipitated with 2 vol. of ethanol and collected by centrifugation. Recovery of 120 n RNA was essentially complete; with increasing size of RNA, elution was progressively less effective. RNA of size greater than 800 n was not eluted. Electro-elution was carried out in 0.5 $\times$ TBE with gel slices in 0.5 ml of 0.5 $\times$ TBE in dialysis tubing. Electrophoresis was carried out at 100 mA (initially 200 V) for 2–3 h. Recovery averaged 66%, but was independent of RNA size.

RNA was fingerprinted by the procedure of Brownlee (1972), essentially as described by Bogenhagen *et al.* (1982). The ribonuclease T1 digests were fractionated by electrophoresis on cellulose acetate strips at pH 3.5, followed by ascending chromatography on polyethyl-enimine-cellulose thin-layer plates (Sybron-Brinkmann) in 1.5 M-ammonium formate, pH 3.5.

Hybridization analysis

DNA was transferred to nitrocellulose membranes (BA 85, Schleicher and Schuell) after agarose-gel electrophoresis of restriction-enzyme-digested DNA ('Southern blots') (Maniatis *et al.*, 1982). Routinely, 5 μ g of genomic DNA or 1 μ g of plasmid DNA (linearized by restriction-enzyme digestion) were used. To obtain 'Dot' blots, 0.5 M-NaOH containing 1 M-NaCl was added to the DNA to a final concentration of 0.3 M-NaOH. After heating at 60 °C for 5 min, an equal volume of 1.8 M-Tris/HCl, pH 6.8, was added; the solution was mixed and applied to nitrocellulose. The nitrocellulose filters were then baked at 80 °C for 2 h.

After wetting in 4 $\times$ SSC, the blots were equilibrated in hybridization solution [4 $\times$ SSC containing 50% (v/v) formamide, 1 mM-EDTA and 0.1% SDS] for a minimum of 2 h at 43 °C in sealed bags. This solution was removed, labelled RNA synthesized *in vitro* in a minimum volume of hybridization solution was added, and the bags resealed. After heating at 80 °C for 5 min, hybridization was carried out at 37 °C or at 43 °C with gentle agitation. Unhybridized RNA was removed under either low-stringency conditions or high-stringency conditions. Under low-stringency conditions, the filters were washed in 2 $\times$ SSC for 20 min three times at room temperature. Under high-stringency conditions, the filters were first washed in 2 $\times$ SSC for 20 min four times at room temperature and then washed in 0.1 $\times$ SSC for 20 min twice at 60 °C.

RESULTS

Transcription of genomic *Xenopus* DNA

Multiple discrete RNA species were synthesized with *Xenopus* erythrocyte DNA as template. The synthesis of all *Xenopus* RNA species was inhibited by α -amanitin at 200 μ g/ml, but not at 1 μ g/ml, indicating that all RNA species were synthesized by RNA polymerase III. Similar results were obtained with *Xenopus* liver and kidney DNA (results not shown). The multiplicity of RNA species was not the result of processing, for in a pulse-chase experiment there was no change in the relative proportion of RNA species upon continued incubation after the addition of a vast excess of unlabelled nucleotides (Fig. 1, lanes a–j). In a parallel experiment with plasmid DNA containing the xlo 5S rRNA gene–pseudogene cluster, similar results were obtained (Fig. 1, lanes k–p). This experiment also indicated that there was relatively little degradation of synthesized RNA.

Various size classes of RNA transcribed from *Xenopus* DNA were eluted from the gel and hybridized to Southern blots of *Hind*III-digested *Xenopus* DNA. (In *Xenopus* the 5S rRNA repeat is largely within a *Hind*III fragment of about 820 bp.) RNA species denoted a, b, c, and d in Fig. 1 have sizes of about 450–600 n, 300–380 n, 170–200 n and 100–120 n respectively. As Fig. 2 shows, RNA species of fraction a did not hybridize to the *Hind*III fragment containing genomic 5S rDNA (arrow), whereas the other three fractions did. However, RNA species b and c (as well as a) did not hybridize to the cloned *Xenopus* 5S rDNA insert in Southern blots of *Hind*III digested pHU1075, suggesting that these RNAs were not transcribed from the 5S gene–pseudogene cluster, but from other DNA sequence(s) of the same size as the *Xenopus* *Hind*III fragment containing the 5S

Fig. 1. Transcription of *Xenopus* erythrocyte DNA and plasmid DNA containing the 5S rRNA gene-pseudogene cluster

RNA was synthesized with 20 μM - ^{32}P GTP. Incubation at 30 °C (*Xenopus* erythrocyte DNA template) for: 20 min (lane a), 30 min (lane b), 60 min (lane c), 75 min (lane h), 90 min (lane i), and 120 min (lane j). Unlabelled GTP was added to 640 μM at 60 min, a sample taken (lane d), and incubation continued for 75 min (lane e), 90 min (lane f), and 120 min (lane g). Incubation at 30 °C (pHU1075 DNA template) for: 30 min (lane k), 90 min (lane o), 120 min (lane p). GTP was added to 640 μM at 60 min, a sample taken (lane l), and incubation continued for 90 min (lane m), and 120 min (lane n). In a parallel experiment with GTP at 640 μM and 5 μCi of ^{32}P GTP, no RNA synthesis was detectable.

ribosomal gene-pseudogene (approx. 820 bp) cluster. In contrast, RNA species d, 100–120 n in size, hybridized to the 5S rDNA insert of pHU1075 DNA, indicating that this RNA contained 5S rDNA transcripts. Since the largest RNA species, a, do not hybridize to the *Hind*III fragment containing this repeat DNA, it is likely that this RNA was transcribed from a second class of repeat DNA.

Transcription of plasmid DNA containing the xlo 5S rRNA gene-pseudogene cluster

Since less than 10% of RNA transcribed from genomic *Xenopus* DNA was 5S rRNA (Fig. 2), transcription of pHU1075 DNA containing the *Xenopus* 5S rRNA gene-pseudogene cluster was examined in greater detail. [This plasmid contains three copies of the 5S gene-pseudogene cluster (xlo 36) inserted into pBR322 at the *Hind*III site in reverse orientation to that of the vector (Federoff & Brown, 1978; Miller *et al.*, 1978) (Fig. 3a). The pseudogene is a duplication of the first 101 n of the 5S gene and is 85% homologous with the 5S gene (Jacq, 1977; Federoff & Brown, 1978; Miller *et al.*, 1978); the

promoter sequence (Sakonju *et al.*, 1980; Bogenhagen *et al.*, 1980) is present, but the normal termination sequence is absent.] Three discrete RNAs were transcribed. The smallest RNA (Fig. 1, d) is 120 n in size and co-migrated with 5S rRNA isolated from rat liver or calf thymus. The largest RNA migrated on 10%-(w/v)-acrylamide gels slightly faster than did the 194 bp DNA marker (marker not shown).

The 120 n RNA hybridized to the 5S rDNA insert in Southern blots of *Hind*III-digested pHU1075 DNA but not to vector DNA (Fig. 3b, lane a). Comparison of fingerprints of this RNA with published fingerprints of *Xenopus laevis* 5S rRNA species (Brown *et al.*, 1977) revealed oligonucleotides expected of transcripts of the *Xenopus laevis* pseudogene as well as an oligonucleotide present in 5S rRNA but not in a putative pseudogene transcript. Pseudogene transcripts presumably terminated in the A1 region at CATTTTCA (Federoff & Brown, 1978).

The largest discrete RNA hybridized to both insert and vector DNA in Southern blots of *Hind*III digested

Fig. 2. Hybridization of multiple discrete RNA species transcribed from *Xenopus* erythrocyte DNA to genomic *Xenopus* DNA and pHU1075 DNA, both digested with *Hind*III

xlo 5S RNA (1500 c.p.m.) was obtained by transcription of pHU1075 DNA. The four groups of RNA transcribed from *Xenopus* erythrocyte DNA used were: (a) 450–600 n (5600 c.p.m.); (b) 300–380 n (3100 c.p.m.); (c) 170–200 n (3700 c.p.m.); (d) 100–120 n (1000 c.p.m.). RNA species were incubated with blots containing: a, genomic *Xenopus* liver and kidney DNA (4.7 μ g); or b, pHU1075 DNA (1.4 μ g). Hybridization was at 37 °C for 2 days (low-stringency wash). Radioautography was for 2 weeks.

pHU1075 (Fig. 3b, lane b) and was not transcribed from plasmid DNA cleaved with *Hind*III or *Eco*R1 (Fig. 3c). Since DNA scission by both these enzymes would prevent read-through transcription into pBR322 of RNA originating from the last 5S rDNA cluster, this RNA represented transcripts initiating in the 5S rRNA pseudogene and terminating in the vector in response to a strong signal GCCTATTTTATAG after an RNA of about 180 n was transcribed.

The intermediate-sized RNA was not characterized. However, its size is consistent with transcription through the normal 5S rRNA termination site, ending at a second signal, CTTTATTTTGC, in the B2 region (Miller *et al.*, 1978).

The *Xenopus* 5S rRNA pseudogene is transcribed by extracts of *Xenopus* oocyte nuclei (Miller & Melton, 1981). These studies indicate that RNA polymerase III in HeLa-cell cytosol extract transcribed both the 5S rRNA pseudogene as well as the 5S rRNA gene in the cloned gene-pseudogene cluster. Comparison of the individual oligonucleotide signal intensities on the radioautographs of the fingerprint suggests that the gene and the pseudogene were transcribed with approximately equal efficiency.

Transcription of genomic bovine DNA

RNA synthesized was heterogeneous in size except for a discrete 120 n RNA (Furth & Su, 1983; Furth, 1985; Fig. 4, lane a).

RNA species of 120 n, transcribed from calf thymus and pTH1 DNA (containing hamster 5S rDNA) were eluted from the gel and digested with ribonuclease T1. In all, 22 different oligonucleotides were found in 120 n RNA transcribed from pTH1, and these oligonucleotides were those expected to be obtained in T1 digests of

hamster 5S rRNA (results not shown). About the same number of oligonucleotides were observed in the T1 digest of bovine 5S-sized RNA; however, most of the large oligonucleotides were dissimilar. The limited number of oligonucleotides present in the bovine 5S-sized RNA suggested that this RNA was relatively homogeneous.

Hybridization of the bovine 5S-sized RNA to a genomic library resulted in hybridization to 90% or more of the plaques, whereas, in a parallel experiment with a duplicate blot, hamster 5S rRNA hybridized to few, if any, plaques (results not shown).

The 120 n RNA also hybridized to the clonal bovine repeat insert in *Bam*HI-digested pPGA7 (cloned bovine repeat) (results not shown). No hybridization to genomic mouse or human DNA was observed, even though hybridization was carried out at low stringency. Thus the transcribed bovine repeat bears little sequence homology with corresponding repeats in human and mouse DNA.

Large (180–650 n) RNA was eluted from the gel and also hybridized to the genomic library. Hybridization to over 90% of the plaques was observed, and the intensity of hybridization to individual plaques was about the same as that of the 120 n bovine RNA, indicating that similar repeats were transcribed. The larger RNA also hybridized strongly to Dot blots of bovine DNA and did not hybridize to mouse or human DNA (results not shown). On hybridization at high stringency to Southern blots of *Bam*HI-digested pPGA7 (cloned bovine repeat) DNA, the larger RNA hybridized to the insert (as did the 120 n RNA). This result indicated that the larger RNA and the 120 n RNA were transcribed from very similar repeats. Transcription of two cloned bovine repeat DNA species resulted not only in 120 n RNA but also in larger RNA

Fig. 3. Transcription of the plasmid DNA (pHU1075) containing the 5S rRNA gene-pseudogene cluster

(a) Diagram of pHU1075 DNA. Top: Relationship of insert to vector. Bottom: Structure of the 5S gene, pseudogene and spacer regions (Federoff & Brown, 1978; Miller *et al.*, 1978). (b) Hybridization of RNA transcribed from pHU1075 DNA to Southern blots of *Hind*III-digested pHU1075 DNA. a, 120 n RNA (4800 c.p.m.); b, large RNA (24000 c.p.m.). Hybridization was at 37 °C for 2 days with low-stringency wash. Radioautography was for 3 days. v, vector; i, insert. (c) Polyacrylamide-gel analysis of RNA transcribed from pHU1075 DNA cleaved with *Hind*III and *Eco*R1. RNA was synthesized with [³²P]GTP and 0.8 µg of DNA in a 50 µl reaction volume. a, pHU1075 DNA, digested with *Hind*III; b, pHU1075 DNA digested with *Eco*R1; c, pHU1075 DNA incubated under identical conditions used for *Eco*R1 digestion, but without enzyme. (The simultaneous addition of restriction enzyme to the synthetic reaction resulted in the pattern seen in lane c.) The location of 5S rRNA is indicated by an arrow.

species of sizes comparable with that seen on transcription of genomic bovine DNA (Fig. 4). This is more readily apparent in gels that were radioautographed for shorter periods of time.

5S rRNA may be transcribed from genomic bovine DNA

RNA 120 n in size is not only transcribed from genomic bovine DNA but is also transcribed from genomic human DNA (Wu, 1978; Furth, 1985). Bovine 120 n RNA hybridized at high stringency to hamster 5S rDNA and to one discrete *Hind*III fragment of genomic bovine DNA (Fig. 5c). Hybridization to a discrete *Xba*I fragment was also observed; hybridization to discrete fragment(s) was not observed when DNA was digested with *Eco*R1, *Bam*HI, *Sma*I, *Taq*I or *Alu*I (results not

shown). Human 120 n RNA also hybridized (very faintly) to this same sized fragment (arrow, Fig. 5a), as did authentic 5S rRNA (Fig. 5b).

The stability of hybridization of bovine 120 nRNA to the bovine *Hind*III fragment was compared with the stability of 5S rRNA-5S rDNA hybrids. The discrete bovine 120 n RNA-bovine DNA hybrid was still observed at 75 °C (in 0.1 × SSC), whereas hybridization (as a smear) of large RNA transcribed from bovine DNA, faintly observed at 65 °C, was gone at 70 °C. In a parallel experiment with 120 n RNA transcribed from cloned hamster 5S rDNA, the hybrid also remained at 75 °C (results not shown).

Just as similar results were obtained on transcription of *Xenopus* erythrocyte, and of liver and kidney DNA

Fig. 4. Polyacrylamide-gel analysis of RNA transcribed from genomic bovine DNA and plasmid DNA species containing bovine repeats

[32 P]UTP was used as label, and 25 μ l reaction volumes were employed. DNA species were as follows: a, Calf thymus (1 μ g); b, Calf thymus (3 μ g); c, pPGA7 (0.4 μ g); d, pPGA7 (1.5 μ g); e, pPGB9 (0.4 μ g); f, pPGB9 (1.5 μ g); g, pHU1075 plasmid containing the xlo 5S rRNA gene-pseudogene cluster (0.4 μ g).

species, similar results were obtained on transcription of bovine DNA obtained from fetal-calf thymus, calf liver and bovine lymphosarcoma (Furth, 1983). The size distribution of RNA species differed from RNA transcribed from a variety of human DNA species (Furth, 1983) and from mouse DNA. RNA transcribed from genomic mouse DNA, either liver or thymus, included not only heterogeneous RNA, approx. 400–600 n, but also a discrete RNA of about 170 n. Both the large RNA and the 170 n RNA were transcribed from mouse Alu-type repeat DNA species (results not shown).

DISCUSSION

Most RNA transcribed from genomic *Xenopus* DNA was transcribed from Alu-type repeat DNA

RNA transcribed from *Xenopus* DNA was observed as a relatively limited number of discrete size classes. Most of these RNA species were transcribed from Alu-type repeats. There are at least two classes of these repeats in the *Xenopus* genome, since the larger RNA species synthesized *in vitro* did not hybridize to the 820 bp *Hind*III fragment.

The smaller RNAs hybridized to a *Hind*III fragment the same size as the fragment containing the 5S rRNA gene-pseudogene cluster. A *Hind*III fragment of about 820 bp contains the *Xenopus* satellite 1 sequence (Lam & Carroll, 1983), which has a highly active RNA polymerase transcription unit (Andrews *et al.*, 1984). The promoter element in satellite 1 DNA is 10–25-fold more efficient than the 5S rRNA gene promoter element (Andrews *et al.*, 1984). The efficiency of this promoter accounts, in part, for the predominance of this RNA over 5S rRNA in synthesized RNA, although there are 25000 5S rRNA genes in *Xenopus laevis* (Peterson *et al.*, 1980). The second repeat containing an RNA polymerase transcription unit has not yet been characterized.

Fig. 5. Hybridization of 120 n RNA to genomic DNA species and plasmid DNA species containing the 5S rRNA gene

Genomic DNA species (5 μ g) and pHU1075 DNA (0.5 μ g) were digested with *Hind*III; pTH1 DNA (0.5 μ g) was digested with *Bam*H1. Filters were washed at high stringency. Radioautography was for 14 days. DNA species were as follows: C, genomic bovine (calf thymus) DNA; H, genomic human (HeLa) DNA; X, genomic *Xenopus laevis* DNA; pTH, pTH1 DNA; pHU, pHU1075 DNA. (a) 120 n RNA transcribed from human (glioblastoma) DNA (1000 c.p.m.); (b) 120 n RNA transcribed from pTH1 DNA (hamster 5S rRNA containing plasmid) (2200 c.p.m.); (c) 120 n RNA transcribed from fetal-calf thymus DNA (3900 c.p.m.).

In contrast with most mammalian Alu-type repeats, *Xenopus* repeats differ in that all or most of the repeats contain a termination signal located at a constant distance downstream from the promoter element.

Transcription of genomic *Xenopus* DNA by RNA polymerase III in HeLa-cell cytosol extract differs from transcription by RNA polymerase III in *Xenopus* extract

Although multiple discrete RNA species were transcribed from *Xenopus laevis* genomic DNA by RNA polymerase III in *Xenopus* oocyte nuclear extract (Bogenhagen *et al.*, 1982), by far the most prominent RNA was 5S rRNA. In contrast, when *Xenopus* DNA was transcribed by RNA polymerase III in HeLa-cell cytosol extract, little 5S rRNA was synthesized (Fig. 1). Results with HeLa extract are probably not due to insufficient TFI_{IIA} (specific 5S gene transcription factor) in the extract, as the 5S rRNA gene in plasmid DNA was efficiently transcribed (Fig. 1), although it is possible that TFI_{IIA} is limiting and binds to genomic *Xenopus* DNA.

A second explanation is that transcription is proportional to the number of genes. There are far more repeat genes than 5S rRNA genes in genomic DNA, and these genes contain a very efficient promoter element (Andrews *et al.*, 1984). If this were the case, one would not expect as much 5S rRNA synthesis from genomic *Xenopus* DNA by *Xenopus* extract. Since large amounts of 5S rRNA are transcribed from *Xenopus* DNA by *Xenopus* extract (Bogenhagen *et al.*, 1982), it is more likely that the results are due to differing properties of the extract. [Approximately equal quantities (by weight) of genomic *Xenopus* DNA and plasmid DNA were used in the transcription experiment. The *Xenopus* genome contains about 3×10^9 bp (Dawid, 1965). At 25000 5S rRNA genes there would be about 9×10^9 5S rRNA genes in 1 μ g, whereas in 1 μ g of pHU1075 DNA there would be 100-fold more (about 7×10^{11}) 5S rRNA genes. Although this would account for the small amount of 5S rRNA transcribed from genomic *Xenopus* DNA, it would not account for the relatively greater transcription of repeat DNA observed on transcription by HeLa extract than on transcription by *Xenopus* extract (Bogenhagen *et al.*, 1982).]

A third possibility is that a factor or factors in an homologous extract restrict transcription of repeat DNA of that species. We have observed that genomic human DNA and Alu-repeat DNA (in plasmid DNA) were poorly transcribed by human (HeLa) extract, whereas the converse was the case with genomic bovine DNA, bovine Alu-type repeat and bovine extract (Furth, 1985). Thus, in the experiments performed by Bogenhagen *et al.* (1982), the *Xenopus* extract could have contained factors repressing transcription of *Xenopus* repeats. This would account for the relatively more efficient transcription of 5S rRNA genes than of repeat DNA.

Transcription of genomic bovine DNA

RNA transcribed from genomic bovine DNA by RNA polymerase III in HeLa cell cytosol extract includes a discrete RNA of about 120 n. Although earlier studies were consistent with this RNA being 5S rRNA in that the nearest-neighbour frequency, NpU, was similar to that of 5S rRNA (Furth & Su, 1983), more of this RNA was synthesized than would be expected from the number (approx. 2000) of 5S rRNA genes in *Bos* (Ackerman *et al.*, 1979). Here we have shown that most of this 120 n

RNA, as well as the larger RNA species, were transcribed from bovine repeat DNA.

There is one major bovine interspersed repeat (Watanabe *et al.*, 1982; Richardson *et al.*, 1986), and more than 10^8 copies of this repeat are present, the same order of magnitude as the number of Alu repeats in human DNA. A number of bovine repeats have been sequenced, and a consensus sequence has been derived (Watanabe *et al.*, 1982). The present studies indicate that there is a subset of bovine repeat DNA that contains a termination signal for RNA polymerase III after an RNA of 120 n is transcribed. Although analysis of the sequenced pPGB9 repeat from which 120 n RNA was transcribed showed no strong termination signal (in the *Xenopus* system) (Bogenhagen & Brown, 1981), the 120 n RNA could have resulted from termination (-CAUU) at the sequence CATTTGTTA, since there is a putative promoter (B Box) (Ciliberto *et al.*, 1983) with the sequence GGTTGCAAAGA an appropriate distance upstream.

Although most Alu-type repeats are not transcribed in most cells, a few examples of repeats transcribed *in vivo* by RNA polymerase III have been reported. An Alu-type repeat is transcribed *in vivo* in rat brain, but not in other tissues of this animal (Sutcliffe *et al.*, 1984). This RNA-polymerase-III transcript appears to be transcribed concomitantly with a protein-coding gene. An analogous phenomenon also occurs in erythroid cells. An Alu repeat family member upstream of the human ϵ -globulin gene is transcribed in association with expression of the ϵ -globulin gene (Allan & Paul, 1984). This is consistent with (although not proof of) this repeat functioning in gene regulation. It may be significant that this repeat is transcribed, *in vitro*, two to three orders of magnitude more efficiently than a neighbouring repeat (Paoella *et al.*, 1983).

7SL RNA contains part of a human Alu-type repeat at the 5' end of the molecule (Ullu *et al.*, 1982) and this RNA is transcribed *in vivo* by RNA polymerase III (Zieve, 1981; Ciliberto *et al.*, 1983). Since sequence homologies exist between repeat elements of diverse species flanking a conserved 7SL sequence, it has been suggested that Alu-type repeat sequences are processed 7SL RNA genes (Ullu & Tschudi, 1984). If this is the case, the repeat 5' to the conserved sequence in 7SL RNA is likely to represent a repeat bearing a promoter active *in vivo*.

It has also been suggested that Alu repeats function in replication, possibly by facilitating entry of DNA polymerase. In experiments in which the components of the DNA polymerase reaction (deoxyribonucleoside triphosphates, one labelled with ^3H , and DNA polymerase- α) were added to a standard reaction, a 2-fold increase in DNA synthesis was observed. There was no evidence of covalent attachment of newly synthesized DNA to RNA, suggesting that if RNA polymerase III is involved in replication, it is by a mechanism other than synthesizing primer RNA (J. J. Furth, unpublished work).

Transcription of 5S rRNA from genomic DNA templates

5S sized RNA, 120 n, transcribed from genomic human DNA hybridized strongly to 5S rDNA (Fig. 5) and to one discrete fragment of *Hind*III-digested bovine DNA. Hybridization to 5S rDNA indicated that the 120 n RNA transcribed from genomic human DNA was largely 5S rRNA. Bovine 120 n RNA also hybridized strongly to 5S

rDNA and to a discrete *Hind*III fragment of bovine DNA (Fig. 5). Both hybrids survived washing at an elevated temperature. Authentic 5S rRNA hybridized to the same size bovine fragment and the hybrid was of the same thermal stability.

The melting temperature, T_m , of a mammalian 5S rRNA duplex (60% GC) is 76 °C in 0.1 × SSC (Marmur & Doty, 1962; Dove & Davidson, 1962), but RNA-DNA hybrids are slightly more stable than the corresponding DNA-DNA duplex (Casey & Davidson, 1977). Persistence of the hybrids at 75 °C indicates the presence of 5S rRNA in the 120 n RNA transcribed from genomic bovine DNA and strongly suggests that bovine 5S rRNA genes are to be found in a discrete *Hind*III fragment.

In these experiments, no differences were observed on hybridization of authentic (hamster) 5S rRNA to Southern blots of plasmid DNA species containing hamster or *Xenopus* 5S rDNAs. Although the sequences of mammalian 5S rRNA genes are very similar (Ackerman *et al.*, 1979; Hart & Folk, 1982; Walker *et al.*, 1975; Erdmann *et al.*, 1985), 14 n are different in *Xenopus* 5S rRNA (Erdmann *et al.*, 1985). However, there is a 20 n stretch of perfect homology, and only about 20 base-paired nucleotides are required for hybridization (Walker, 1969). The ratios of 5S rRNA genes in the blots of pHU1075, pTH1, genomic *Xenopus* DNA and mammalian DNA were approx. 120:42:12:1. The absence of hybridization of both bovine and human 120 n RNA to the 5S rDNA-containing fragment of *Hind*III-digested genomic *Xenopus* DNA may have been a consequence of the variation in size of the *Xenopus* spacer and the presence of the *Xenopus* 5S rRNA pseudogene. *Xenopus* spacer sequences vary up to 200 bp in size (Federoff & Brown, 1978; Miller *et al.*, 1978), and this would result in a weaker signal on radioautography than would be predicted from the number of 5S rRNA genes in the DNA. There is a 21% mismatch and no 20 bp homologous region between mammalian 5S rRNA and the *Xenopus* 5S pseudogene. Therefore, whereas 5S rRNA would hybridize to pseudogene DNA in 50% formamide/4 × SSC at 43 °C, this RNA would be lost on washing in 0.1 × SSC at 60 °C.

Transcription *in vitro* in a heterologous system

Although differences exist between homologous and heterologous transcription systems *in vitro* [Furth, 1985; Shaack & Söll, 1985 (and references cited therein)], the basic apparatus appears to be similar, and accessory transcription factors are effective in heterologous systems. A notable difference is the decreased transcription efficiency of Alu-type repeat DNA in the homologous system (Furth, 1985). However, 5S rRNA appears to be transcribed by human cytosol extract from both genomic bovine and genomic human DNA. Synthesis of bovine 5S rRNA was obscured by the concomitant transcription of bovine repeat DNA, which fortuitously resulted in transcription of RNA of a similar size.

The amounts of repeat RNA (including 120 n RNA) and 5S rRNA synthesized were roughly consistent with the relative amounts of Alu-type repeat DNA and 5S rDNA present in the bovine genome (Watanabe *et al.*, 1982; Richardson *et al.*, 1986; Ackerman *et al.*, 1979). No transcription of RNA the size of tRNA or tRNA precursor was observed. However, by using a homologous system and substrates of higher specific radioactivity than those used in these experiments, it should be possible to

probe genomic DNA to assess transcription of tRNA genes as well as to seek out as-yet-unidentified polymerase III transcription units. It may also be possible to delineate repeat DNA promoters active *in vivo* by transcribing chromatin of different tissues, since RNA synthesized *in vitro* from chromatin accurately reflects the situation *in vivo* (Bogenhagen *et al.*, 1982).

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