Transfection of mouse ribosomal DNA into rat cells: faithful transcription and processing

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ABSTRACT

Truncated mouse ribosomal DNA (rDNA) genes were stably incorporated into rat HTC-5 cells by DNA-mediated cell transfection techniques. The mouse rDNA genes were accurately transcribed in these rat cells indicating that there is no absolute species specificity of rDNA transcription between mouse and rat. No more than 170 nucleotides of the 5' nontranscribed spacer was required for the accurate initiation of mouse rDNA transcription in rat cells. Further, the mouse transcripts were accurately cleaved at the 5' end of the 18S rRNA sequence, even though these transcripts contained neither the 3' end of mouse 18S rRNA nor any other downstream mouse sequences. Thus, cleavage at the 5' end of 18S rRNA is not dependent on long range interactions involving these downstream sequences.

INTRODUCTION

In mammalian cells, mature 18S, 5.8S and 28S ribosomal RNAs (rRNAs) are generated by processing of a 45S precursor (pre-rRNA) (1,2). The analogous pre-rRNA in *E. coli* is initially cleaved within two exceptionally stable stems formed by base pairing of the spacer sequences that flank each of the mature rRNAs (3,4). In mammals and other eukaryotes, such exceptionally stable stems formed by base pairing of spacer sequences flanking mature rRNAs are not possible (5-10). Furthermore, the initial cleavages of eukaryotic pre-rRNA usually occur at the ends of mature rRNAs rather than within spacer sequences as is the case in *E. coli*. These results suggest that the mechanisms of pre-rRNA processing in eukaryotes are quite different from those in *E. coli*. However, less stable stems formed by long range base pairing of spacer or mature rRNA sequences are possible in eukaryotes. It is not known whether these potential long range interactions help signal pre-rRNA processing in eukaryotes.

The sequence requirements for the transcription of mammalian rDNA in vitro have been analyzed by measuring the transcription of modified templates in cell extracts (11-19). Only 40 nucleotides of the 5' nontranscribed spacer and 52 nucleotides of transcribed spacer sequences are required for accurate initiation of mouse rDNA transcription in vitro (17-19). However, recent experiments suggests that the sequence requirements for mouse rDNA transcription in vivo may be different (20). The transcription of Xenopus laevis rDNA is stimulated by enhancer-like repeats located several kb upstream from the transcription start site (21-22). Although enhancer-like sequences have not been identified in the sequenced region of the mouse 5' nontranscribed spacer (20, 23-25), it is possible that transcription stimulatory elements exist in upstream unsequenced regions.

One approach to these questions concerning the processing and transcription of mammalian pre-rRNA and rDNA is to measure the expression of modified rDNA genes introduced into living cells of another mammalian species. However, the transcription of rDNA appears to be species specific (26-30), at least between mouse and human cells, and it is not known whether pre-rRNA processing is also species specific. In this paper, we show that mouse rDNA incorporated into rat cells is accurately transcribed, and that mouse pre-rRNA is faithfully processed in rat cells. Our results indicate that cleavage of mouse pre-rRNA at the 5' end of the 18S rRNA sequence is not dependent on long range interactions with sequences at the 3' end of 18S rRNA or any other downstream mouse rDNA sequences. Furthermore, accurate initiation of mouse rDNA transcription in rat cells requires no more than 170 nucleotides of the mouse 5' nontranscribed spacer.

MATERIALS AND METHODS

Subcloning of mouse rDNA. The subclone pMr974 was generated by cloning the 14 kb Eco R1 insert of lamda gtWes Mr974 (clone 3) (31) into the Eco R1 site of the plasmid pUC-8 (32). The subclone pA20 was generated by digesting pMr974 to completion with Eco R1 but only partially with Sal 1 followed by ligation into Eco R1-Sal 1 digested pUC-9 (32). The clone that contained sequences from

the Eco R1 site in 18S rRNA to the Sal 1 site 170 nucleotides upstream from the transcription start site (figure 1) was selected.

DNA-mediated cell transfection. Rat HTC-5 cells were plated at a density of 1×10^6 cells/100mm culture dish in D-MEM medium containing 5% horse serum. Twenty four hours later the cultures were incubated with a calcium phosphate precipitate that contained 25-40 ug of pMr974 or pA20 and 1 ug of pSV2-neo (33). The calcium phosphate precipitates were formed by dissolving the DNA in 1 ml of a buffer containing 0.020 M HEPES, pH 6.95, 0.136 M NaCl, 0.5 mM $\text{Na}_2\text{HPO}_4 \cdot 7 \text{H}_2\text{O}$, and then adding 100 ul of 2.5 M CaCl_2 in small drops around the sides of the tube followed by vortexing (34). After 20 min of incubation at room temperature, the precipitates were added to the cells. After 24 hr, the transfected cells were rinsed with media and split 1 to 3. The antibiotic G-418 was added the following day to a final concentration of 400 ug/ml. Cultures were subsequently fed with fresh G-418 media every third day. G-418 resistant clones were isolated approximately two weeks after transfection by trypsinization in cloning cylinders.

Isolation of DNA and RNA. Nucleic acids were isolated from G-418 resistant rat clones or nontransfected control rat cells by SDS-proteinase K, phenol/chloroform extraction (8). Cells were harvested in 0.25 M NaCl, 0.02 M Tris-HCl, pH 7.4, 0.005 M EDTA, 1% SDS and 1 mg/ml proteinase K, incubated at 37°C for 30 min, phenol-chloroform extracted once, chloroform extracted once and ethanol precipitated. DNA and low molecular weight RNAs were separated from high molecular weight RNA by rinsing ethanol precipitated nucleic acids with 2 M LiCl_2 (35). This rinse contains DNA and low molecular weight RNAs and was diluted with an equal volume of water and precipitated with 0.6 volumes of isopropanol. Low molecular weight RNAs were separated from DNA by digestion with 10 ug/ml of RNase A followed by phenol-chloroform extraction.

Hybridization analyses. Cellular RNA from nontransfected rat HTC-5 cells and each of the G-418 resistant clones was glyoxylated, fractionated by agarose gel electrophoresis, and blotted to nitrocellulose paper (36,37). This northern blot was

baked at 80° C for 2 hr in a vacuum oven and placed in 30 mM Tris-HCL, pH 8.0, at 95° C (34). The blot was removed from this solution after it cooled to room temperature. Both northern and Southern (38) blots were prehybridized at 45° C for 4-8 hr in 50% formamide, 5X SSC, 0.2% SDS, 0.02 M Na phosphate, pH 6.8, 0.002 M EDTA, 150 ug/ml denatured calf thymus DNA and 5X Denhardt's solution. The blots were hybridized for 12-24 hr at 53° C in prehybridization mix containing 10% dextran sulfate and 3 ng/ml of nick-translated probe. The blots were rinsed as described except that the last rinse was performed at 65° C (8,39). S1 nuclease mapping was performed exactly as described previously for strand separated probes (10,40).

RESULTS

Two plasmids containing truncated mouse rDNA genes were transfected into rat HTC-5 cells. One plasmid (pMr974) contains 8.2 kb of the 5' nontranscribed spacer and 5.8 kb of transcribed sequences, including the entire external transcribed spacer and the first four-fifths of the 18S rDNA sequence (figure 1). The other plasmid (pA20) is identical to pMr974 except it contains only 170 nucleotides of the 5' nontranscribed spacer (figure 1). These truncated mouse rDNA genes were introduced into rat cells by DNA-mediated cell transfection along with small amounts of the plasmid pSV2-neo which contains a dominant selectable marker coding for resistance to the antibiotic G-418 (33,34). Following transfection, the rat cells were selected for the expression of the selectable marker by incubating the culture in G-418. Ten G-418 resistant clones were isolated from the transfection with pMr974 and twelve were isolated from the transfection with pA20.

The RNAs isolated from these clones were examined by northern blot analysis to determine if they also express mouse rRNA sequences. The hybridization probe was a gel-isolated restriction fragment from the mouse external transcribed spacer which does not cross hybridize with rat sequences (figure 1, lane 1). Three of the ten colonies from the pMr974 transfection and one of the clones from the pA20 transfection express mouse rRNA sequences. The three clones from the pMr974 transfection are probably independent clones, although we cannot rigorously

Figure 1. Northern analysis of RNA isolated from the rat transformants. G-418 resistant clones. RNA was isolated from the various transformed rat clones, denatured with glyoxal, fractionated on a 1% agarose gel and blotted to nitrocellulose paper (36,37). The isolated insert from p4 (8) was used as the hybridization probe. This fragment is located approximately 319-900 nucleotides upstream from the 5' end of the 18S rRNA sequence (8,9). Lane 1 shows hybridization with 8 ug of control rat RNA. Lanes 2-4 show hybridization with 8 ug of RNA isolated from the pMr974 clones (pMr974 clones C, B, and A, respectively). Lane 5 shows hybridization with 8 ug of RNA isolated from the pA20 clone. Lane 6 shows hybridization with 0.5 ug of control mouse RNA. This figure is a composite of different northern blots. Lanes 1-5 were exposed to x-ray film approximately 5-10 times longer than lane 6. The diagram shows the structure of the mouse rDNA within the plasmids pMr974 and pA20 rDNA used in the transfections. NTS is the nontranscribed spacer. TS is the transcription start site. ETS is the external transcribed spacer. ITS 1 is the internal transcribed spacer 1, and ITS 2 is the internal transcribed spacer 2.

Figure 2. Southern analysis of DNA isolated from the rat transformants (38). DNA was digested with Pst 1, fractionated by agarose gel electrophoresis, blotted to nitrocellulose and hybridized to a mouse external transcribed spacer probe (isolated insert from p4-see legend to figure 1). In this case hybridization was performed at 56° C. Pst 1 cuts mouse genomic DNA 1.7 kb upstream and 0.7 kb downstream from the 5' end of the 18S rRNA sequence (31). Lane 1: 0.5 ug of mouse DNA. Lane 2: 0.1 ug of mouse DNA. Lane 3: 0.05 ug of mouse DNA. Lane 4: 10 ug of rat DNA. Lanes 5-7: 10 ug of DNA from the three pMr974 transformants that express mouse rRNA. Lanes 8-9: 10 ug of DNA from the pMr974 clones that do not express mouse rRNA. Lane 10: 10 ug of DNA from a pA20 clone that does not express mouse rRNA. Lane 11: 10 ug of DNA from the pA20 clone that expresses mouse rRNA.

eliminate the possibility that they are derivatives of the same transfection event. Figure 1 shows that the predominate transcript in each of the pMr974 clones is approximately 7 kb in length, although smaller mouse transcripts are present in reduced amounts (figure 1). The predominate transcript in the pA20 clone is slightly larger than mouse or rat 45S pre-rRNA (figure 1).

In order to estimate the number of mouse genes integrated into the transformed rat clones, genomic DNA from the transformed rat clones was analyzed by Southern blot hybridization techniques. The DNA was cut with Pst 1, which generates a 2.4 kb mouse rDNA fragment that contains external transcribed spacer sequences and 18S rDNA sequences (31). The blot was hybridized with a gel-isolated, mouse specific external

transcribed spacer probe. The 2.4 kb mouse rDNA fragment was detected in the rat clones that expressed mouse rRNA (figure 2, lanes 5-7,10). Based on the comparison of these hybridization signals with those from known amounts of mouse DNA (figure 2, lanes 1-3), and assuming 200 rDNA genes/haploid mouse genome (41), we estimate that the pMr974 clones contain 1-3 copies and the pA20 clone contains approximately 10 copies of the mouse rDNA gene. Although the mouse probe does hybridize to a small extent to a 2.4 kb band in DNA isolated from nontransfected rat cells (figure 2, lane 4) and from clones that do not express mouse rRNA sequences (figure 2, lanes 8-10), this small amount of cross hybridization does not interfere with our estimates of the number of mouse rDNA genes integrated into the rat cell genomes. The clones that do not express mouse rRNA sequences probably either do not contain the mouse rDNA sequences (figure 2, lane 10) or probably contain rearranged mouse rDNA (figure 2, lane 8).

To determine if the mouse rRNA expressed in these clones initiates from the known mouse transcription start site (11,12), the 5' ends of the mouse transcripts were analyzed by S1 nuclease mapping. RNA isolated from the transformants was hybridized to a 5' end labeled mouse hybridization probe which overlaps the mouse transcription start site and does not cross hybridize with rat sequences (figure 3, lane 6). The resulting hybrids were digested with S1 nuclease, and the protected fragments were fractionated by polyacrylamide-urea gel electrophoresis. Figure 3 shows that RNA isolated from the pMr974 or pA20 transformants protects 155 nucleotides of the hybridization probe. This is the same length protected by control mouse RNA and is the length expected for RNAs initiating at the known mouse transcription start site (11,12). Therefore, mouse rDNA transcription is faithfully initiated in these rat cells. Furthermore, the pA20 rDNA is accurately initiated even though it contains only 170 nucleotides of the 5' nontranscribed spacer DNA. This indicates that sequences upstream from -170 are not essential for accurate initiation of mouse rDNA transcription in rat cells.

The steady state levels of the mouse rRNAs expressed in the rat transformants were estimated by comparing the hybridization signals obtained using control mouse RNA to those obtained using

Figure 3. S1 nuclease mapping of the mouse transcription start site. The hybridization probe used in this experiment was a strand separated mouse Sal 1-Sma 1 restriction fragment that begins 168 nucleotides upstream from the transcription start site and ends 155 nucleotides downstream from the start site (11,12,23,24). Lane 1: Hae III digested OX174 markers. Lanes 2-4: S1 mapping of 10 ug of RNA isolated from the pMr974 clones (B, A, and C, respectively) that express mouse rRNA. Lane 5: S1 mapping of 20 ug of RNA isolated from the pA20 clone that expresses mouse rRNA. Lane 6: S1 mapping of 10 ug of RNA isolated from nontransfected rat cells. Lanes 7-9: S1 mapping of 0.075, 0.23 and 0.38 ug of mouse RNA, respectively.

RNA isolated from the rat transformants (from figure 3). The hybridization signal from 10-20 ug of RNA isolated from the transformants cells is 1/3 to 1/10 the signal detected using 0.075 ug of mouse RNA (compare lane 2-5 to lane 7 in figure 3). Thus, the mouse transcripts in both the pMr974 and the pA20 transformants accumulate to about 1/400-1/1300 the level observed for mouse transcripts in mouse cells. Taking into consideration that the pA20 transformant contains 10 copies of rDNA per haploid genome, and that mouse cells contain 200 copies of rDNA per haploid genome (41), then the steady state level of mouse RNA in the pA20 transformant is about 1/20 to 1/65 that in mouse cells on a per gene basis. In contrast, the steady state amount of mouse rRNA in the pMr974 transformants which contain 1-3 copies of mouse rDNA is about 1/2 to 1/6.5 that in mouse cells on a per

gene basis.

The rates of transcription of these truncated mouse genes in rat cells can be estimated from the steady state levels and stabilities of the expressed mouse rRNA. The stability of the truncated mouse rRNA transcripts in rat cells is unknown. However, if these mouse transcripts are equally or less stable in rat cells as compared to mouse cells, then the mouse rDNA in the pMr974 transformants would be transcribed nearly as efficiently ($1/2$ to $1/6.5$) as mouse rDNA in mouse cells. In contrast, mouse rDNA in the pA20 transformant would be transcribed 1-2 orders of magnitude less efficiently.

The mouse transcripts expressed in a pMr974 clone were examined to determine whether they are accurately processed at the 5' end of the 18S rRNA sequence. Clone Mr974A was used for this experiment, since this clone showed the highest level of mouse rRNA expression. We have shown previously that cleavage at this site generates the mature 5' end of 18S rRNA and an external transcribed spacer molecule whose 3' end abutts the 5' end of 18S rRNA (8,10). S1 nuclease mapping can be used to detect small amounts of the cleaved mouse external transcribed spacer molecules in a large background of rat rRNA because these external transcribed spacer sequences are highly divergent. The S1 nuclease mapping probe is a 3' end labeled, strand separated, 419 nucleotide DNA which overlaps the 5' end of the 18S rRNA sequence and contains 220 nucleotides of the external transcribed spacer sequence and 200 nucleotides of 18S rRNA sequence. Consistent with our previous experiments, total mouse RNA contains processed external transcribed spacer molecules that protect 220 nucleotides of the probe (figure 4, lane 1). These correspond to external transcribed spacer molecules whose 3' ends abutt the 5' end of the mouse 18S rRNA sequence. Gel isolated 45S pre-rRNA protects the full length of the probe, as expected for molecules that are uncleaved at this site. Hybridization to RNA isolated from the pMr974 transformant also generates the 220 nucleotide fragment that corresponds to the cleaved external transcribed spacer molecules (figure 4, lane 4). Densitometer tracings of the data in figure 4, as well as other autoradiographs, indicate that the fraction of mouse pre-rRNA

Figure 4. S1 nuclease mapping of the cleavage site located at the 5' end of 18S rRNA. The hybridization probe was a 3' end labeled, strand separated 419 nucleotide *Ava* 1 restriction fragment that contains 220 nucleotides of the external transcribed spacer and the first 200 nucleotides of the 18S rRNA sequence (9,10). Lane 1: S1 mapping of 0.2 ug of total mouse RNA. Lane 2: S1 mapping of approximately 50 ng of gel-isolated mouse 45S pre-rRNA. Lane 3: S1 mapping of approximately 50 ng of gel-isolated mouse 45S pre-rRNA plus 10 ug of RNA isolated from untransfected rat cells. Lane 4: S1 mapping of 10 ug of RNA isolated from Mr974 clone A. Lane 5: S1 mapping of 10 ug of RNA isolated from untransfected rat cells.

molecules cleaved at this site is about one third less in the transformant rRNA than in mouse rRNA. These results suggest that a portion of the mouse rRNA expressed in rat cells is accurately cleaved at the 5' end of the 18S rRNA sequence.

A potential S1 mapping artifact in this experiment is eliminated by the following reconstruction experiment. In order to detect the mouse rRNA sequences in the RNA isolated from the transformants, relatively large amounts of the transformant RNA are used in these hybridizations. Under these hybridization conditions, the 5' end of each probe molecule should hybridize to the abundant rat 18S rRNA sequences, because mouse and rat 18S rRNA sequences are virtually identical (9). An unprocessed mouse molecule could then hybridize to the 3' end of the probe. The junction of these two molecules on the probe could serve as a substrate for S1 nuclease. Cleavage at this site would generate a 220 nucleotide fragment, artifactually suggesting the presence of cleaved mouse external transcribed spacer molecules. If such an artifact is occurring in our experiments, then S1 nuclease mapping of a gel-isolated, mouse 45S pre-rRNA (uncleaved

molecule) in the presence of 10 ug of rat RNA should produce the 220 nucleotide fragment. Figure 4 (lane 3) shows that S1 nuclease mapping of mouse 45S pre-rRNA plus 10 ug of rat RNA does not generate the 220 nucleotide fragment. However, the rat RNA does appear to compete for the probe because the signal generated in this reconstruction is reproducably less than when the probe is hybridized to mouse 45S alone (figure 4, lanes 2 and 3). Despite this, these experiments clearly indicate that the band indicative of accurate processing is not artifactually generated in our hybridizations. This may be because the unprocessed RNA molecules displace the 18S rRNA from the probe as has been suggested in other cases (11).

It is not possible to assay for the 5' end of the truncated mouse 18S rRNA sequence that is generated by cleavage at this site for several reasons. The first is that mouse and rat 18S rRNA sequences cannot be easily distinguished because they are virtually identical. Further, it is not possible to assay for the 5' end of mouse 18S rRNA by extension of a primer complementary to the vector sequences that are probably located at the 3' end of the transcript (see discussion). This is because 18S rRNA contains several stop signals for reverse transcriptase (data not shown). Further, S1 nuclease mapping of untransfected rat RNA using a probe whose 5' end was located in vector sequences always produced a small signal. Thus, it was not possible to use these methods to assay for the presence of small amounts of mouse sequences in a large background of rat sequences.

DISCUSSION

Our results indicate that mouse rDNA introduced into rat cells is accurately transcribed and that the expressed mouse rRNA is accurately cleaved at the 5' end of the 18S rRNA sequence. Thus, the transcription of mouse rDNA and the processing of mouse pre-rRNA can be examined by introducing modified mouse genes into rat cells and determining the amount and structure of the mouse transcripts produced. The mouse transcripts can be distinguished from rat RNA using assays that take advantage of the sequence divergence of their transcribed spacers. In the experiments

reported here, we show that no more than 170 nucleotides of the 5' nontranscribed spacer are required for the accurate initiation of mouse rDNA transcription in rat cells. The pMr974 rat clones whose mouse rDNA contains 8.2 kb of 5' nontranscribed spacer accumulate mouse pre-rRNA to a level nearly as high (about 1/2 to 1/6.5) on a per gene basis as mouse pre-rRNA accumulates in mouse cells. However, the pA20 clone whose mouse rDNA contains only 170 nucleotides of nontranscribed spacer sequences accumulates about an order of magnitude less mouse pre-rRNA on a per gene basis than do the pMr974 transformants. Although our experiments show that rat cells can accurately transcribe mouse rDNA and process mouse rRNA, we have not isolated enough clones to determine whether the difference between the pMr974 and pA20 transformants reflects a significant difference in the capacity of their mouse rDNA templates to support rDNA transcription. If such a difference exists, the sequences stimulating rDNA transcription in the pMr974 transformants are probably located further than 1.9 kb upstream from the transcription start site, because a previous experiment suggests that nucleotides from -170 to -1900 do not stimulate rDNA transcription in vivo (21).

The mouse rRNA expressed in rat cells was accurately cleaved at the 5' end of the 18S rRNA sequence, although the introduced rDNA did not contain the 3' end of the mouse 18S rRNA sequence or any other downstream mouse sequences. Thus, this cleavage is not dependent on any interactions involving mouse sequences downstream from the 3' end of 18S rRNA. Of course, our data do not eliminate the possibility that this cleavage is directed by intermolecular interactions involving rat rRNA sequences located downstream from the 3' end of rat 18S rRNA. However, these results together with previous experiments (8-10) tend not to support models for mouse pre-rRNA processing based on the E. coli paradigm of long range interactions between the spacer sequences that flank mature rRNAs. Although the signals that actually direct pre-rRNA cleavage in eukaryotes are unknown, these may involve only sequences immediately surrounding the cleavage site, most likely the mature rRNA sequences, as these are evolutionarily conserved.

The predominant mouse transcript in each of these rat

transformants is a discrete size and is longer than the transcribed portion of the truncated genes introduced into rat cells. Since the transcripts initiate at the mouse transcription start site, the extra sequences must be located at the 3' end of the transcripts. The discrete size of these transcripts could be due to RNA polymerase terminating at a specific site in the downstream sequences or due to the instability of sequences at the 3' end of the transcript. Further, we do not know what sequences are located at the 3' end of these mouse transcripts, although vector sequences are a likely candidate. However, it seems safe to assume that the corresponding rat rRNA sequences are not located at the 3' end of the mouse transcripts, at least in the pMr974 clones. This would require that the mouse gene integrate into the corresponding location of the rat rDNA gene and would result in the production of a 45S sized transcript. However, the size of the transcript in the pMr974 transformants is only 7 kb in length, approximately one half the length of 45S pre-rRNA. Such a possibility cannot be eliminated for the rDNA in the pA20 clone, because the RNA produced by this clone is just slightly larger than 45S pre-rRNA.

Both rDNA transcription and pre-rRNA processing occur in nucleoli. However, it is not known whether it is obligatory for these events to occur within a nucleolus. Further, it is not known how nucleoli are formed; whether they simply self-assemble around active rDNA genes, or whether there is some signal in rDNA that directs it to nucleoli, for example. Analysis of the intracellular location of these and other stably integrated mouse rDNA genes might help answer these questions.

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