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Transcriptional measurements of mouse repeated DNA sequences

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ABSTRACT

We have carried out transcriptional measurements on several families of repeated sequences to define their expression in mouse cells. The majority of Alu family transcripts result from read-through from adjacent structural gene promoters while 20% are discrete RNA polymerase III products. Alu repeat members show preferential orientation within RNA polymerase II transcription units as evidenced by asymmetric representation of the complementary strands of the Alu family in hnRNA. We assessed whether 3 non-Alu repeated sequence families had their own promoters by strand symmetry measurements and size distribution analysis of repeat-homologous newly synthesized nuclear RNA. Transcription homologous to the R family is totally symmetric and is likely due to read-through from adjacent structural gene promoters. LLRep1 and Bam5 repeats, in contrast, exhibit consistent strand asymmetry which is suggestive that at least some members may be transcribed by their own promoters. Among 3 mouse tissues and 1 cultured cell line analyzed, no quantitative variation in the expression of any of these sequences was observed.

INTRODUCTION

Eukaryotic genomes contain repeated as well as unique DNA sequences. In mammals, the repetitive sequences constitute 10-30% of the genome (1). A single sequence family, the Alu family, has been described as a 300 base pair sequence in humans with 300,000 to 500,000 copies accounting for 6-9% of the total genome (2). An analogous family is found in rodent genomes, with about 75% homology to the human family (3). In addition to the Alu family, there are several other families of interspersed repeated DNA sequences that have been described in mouse and human genomes (4-10). The best studied of these is a long family referred to as KpnI in human and MIF-1 or BamHI in mouse (7,9). At full length, this family is about 6.4 kb, but many of the members are truncated to varying degrees (11). Other mammalian repeat families have been described which range in copy number from 200-40,000 (5,8).
The function(s) of these repeated sequences are largely unknown, although several hypotheses have been advanced. Repeated sequences may function in the

regulation of gene expression (12) or in transposition events (3). Alternatively, repeated sequences may have no function, but may be transcribed fortuitously and dispersed through the genome by the formation of RNA intermediates such as those seen for the processed pseudogenes (13,14). Assessment of the transcriptional properties of repeated sequence families is necessary in evaluating their possible functions since many of the functions would require their transcription. In the sea urchin, the patterns of expression of 9 different repeated sequence families were shown to vary during the course of development (15,16). mRNAs which are coordinately regulated during the differentiation of Dictyostelium were shown to share homology to one strand of a repeated DNA sequence at their 5' ends (17). More recently, it has been suggested that mammalian repeated DNA sequences are expressed in tissue-specific (18,19) and transformation-specific fashions (20).

Previous studies have demonstrated transcription of many rodent and human Alu family members by RNA polymerase III in vitro (21,22). These transcripts are of discrete size, usually less than 600 nucleotides (23). Most in vivo rodent Alu RNA polymerase III transcription results in a single transcript, the 4.5S RNA (24). This molecule binds reversibly to poly(A)$^+$ RNA, suggesting a possible function in mRNA metabolism. A portion of the RNA component of the signal recognition particle shares homology with the Alu family (25). The presence of Alu sequences in the intervening sequences and untranslated regions of a number of structural genes also contributes to the observed expression of the Alu family (26,27). Alu sequences are thus transcribed from one strand by RNA polymerase III from internal recognition sequences and from both strands by RNA polymerase II as read-through from adjacent structural gene promoters. In contrast to the Alu sequences, no RNA polymerase III transcriptional activity has been associated with several non-Alu repeated sequence families (5,8). It is not known whether non-Alu repeated DNA sequences are transcribed by read-through from structural genes or by their own promoters.

In order to distinguish the several proposed functions for repeated DNA sequences, we have characterized the nature and quantity of transcripts homologous to several murine repeated sequence families. We have determined what proportion of Alu-homologous transcription is attributable to RNA polymerase II versus III. We have also examined the orientation of Alu sequences within polymerase II transcription units by asking whether both strands of the DNA sequence are equally represented in the transcripts. Finding a preferred, non-random orientation may imply a function for the Alu

sequences in hnRNA. The non-Alu repeated sequence families LLRep1, Bam5 and R were similarly studied to quantitate the RNA polymerase II-dependent transcription homologous to each strand of these repeated DNA sequence families. (Bam5 and R are each subsets of the longer MIF-1 family). This type of analysis would identify preferred orientation of a sequence contained wthin structural gene transcription units, or, in the case of transcripts derived from only one strand, might imply transcription of the repeated sequence from its own promoter. Finally, we compared the transcriptional properties of these repeated sequence families in 3 mouse tissues and a cultured cell line to determine whether they were differentially regulated.

MATERIALS AND METHODS:

Repetitive sequence probes

Repetitive sequence probes were subcloned from either mouse λ genomic clones (LLRep1, (5); Bam5, (10); R1/R2, (28)) or from cDNA clones (type II Alu; (22)) into the single-stranded phages M13mp8 or M13mp9 in order to generate probes representing the opposite strands of each repetitive sequence family.

Dot Blots

Filters with DNA samples from repeated sequence families were prepared by boiling 2.5 μg of single stranded M13 cloned DNA or 5 μg of pBR322 cloned DNA in 0.1N NaOH, 2M NaCl for 2 minutes. Samples were then spotted onto Gene Screen (New England Nuclear) or nitrocellulose filters. The filters were washed in 2XSSC and baked in a vacuum oven at 80°C for 2 hours prior to hybridization.

Labelling of DNA probes

Double stranded DNA probes were radiolabelled by nick translation as described (29). Radioactively labelled single-stranded M13 probes were prepared as described (30) using a hybridization primer purchased from P-L Biochemicals.

Hybridization

Hybridizations were carried out at 65°C in 5XSSC, 1X Denhardt's, 10mM $NaPO_4$ pH7.4, 50 μg/ml denatured salmon sperm DNA for 16-20 hours. M13 and nick translated probes were included at 2 X 10^5 cpm/ml; kinased RNA and nuclear run-off transcripts were included at 10^6 cpm/ml with the addition of 200 μg/ml yeast tRNA in addition to the denatured salmon sperm DNA. Filters were prehybridized in the above buffer without radioactive probe for 2-6 hours. After hybridization, filters were washed in 2XSSC, 0.2% SDS at 65°C

for 2-4 hours.

RNA Isolation

Total RNA was isolated from the RF strain of mice and from a cultured mouse hepatoma cell line (128 Hpt) by the guanidine-HCl method (31).

Radioactive labelling of RNA

Nuclear run-off transcription was carried out as previously described (5). The assays were carried out on nuclei isolated from mouse brain, liver, kidney and hepatoma cells. Assays were conducted in the absence and presence of the RNA polymerase inhibitor, α-amanitin (Boehringer-Mannheim). Following labelling and RNA isolation, the RNA was TCA-precipitated and collected on nitrocellulose filters prior to hybridization to reduce background (32).

In vivo labelled nuclear RNA was prepared by labelling $2x10^8$ mouse hepatoma cells with ^{3}H-uridine at 0.25 mCi/ml for 10 minutes. Nuclear RNA was isolated, heated in 50% formamide at 60°C for 1 minute, and the RNA species separated by size on a 15-30% sucrose gradient in .05M NaCl, .01M Tris pH7.4, 0.2% SDS. The gradients were centrifuged in an SW41 rotor at 24,000 rpm for 19 hours. Fractions were collected and aliquots counted by TCA precipitation. Aliquots of pooled fractions were electrophoresed on formaldehyde gels followed by blotting onto Gene Screen. Fluorography was carried out by treating the blot with En^{3}Hance spray (New England Nuclear) and exposed to X-ray film.

Measurement of Strand Representation of Nuclear RNA

Radioactively labelled nuclear RNA (in vivo and in vitro) was hybridized to complementary DNA strands of repeated sequence families loaded in excess onto nitrocellulose filters. Complementary strands were obtained by cloning into M13 vectors. The amount of hybridization was quantitated by scintillation counting (in vivo labelled RNA) and densitometry tracing (in vitro labelled RNA).

RESULTS

RNA Polymerase II and III Transcription of Alu Sequences

It is operationally difficult to identify the transcription product of any one Alu family member in vivo due to the high copy number and high degree of sequence homology seen among members. In addition, transcription of the Alu family is complex since members can be transcribed by both RNA polymerases II and III. The relative amounts of RNA polymerase II and III transcription of Alu sequences were assessed using in vivo pulse-labelled hepatoma cell nuclear RNA which was size fractionated on a sucrose gradient. Size

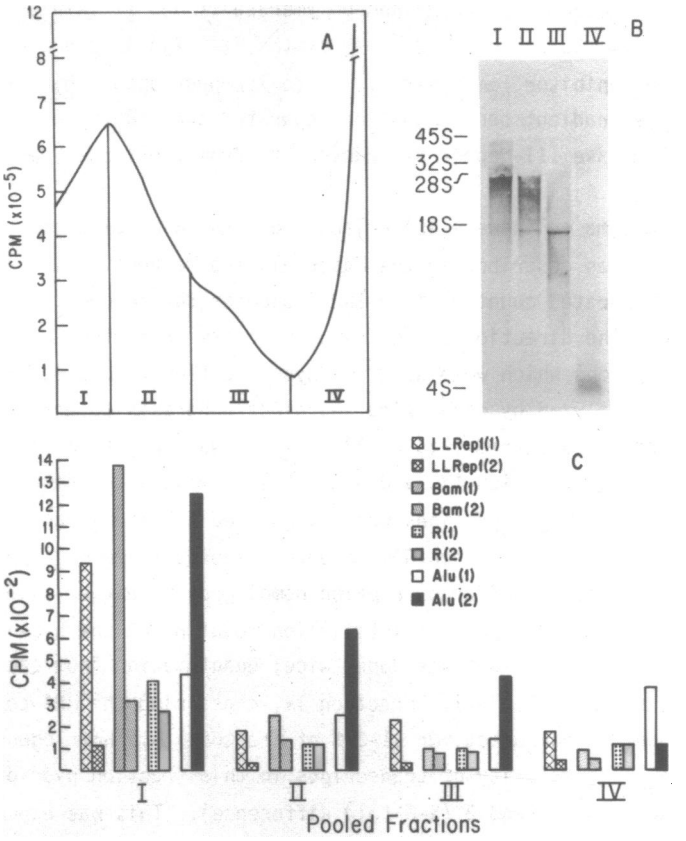

Figure 1: Quantitation of in vivo Labelled Repetitive Sequence Transcripts in Nuclear RNA.

Mouse hepatoma cells were pulse labelled for 10 minutes with ^{3}H uridine and nuclear RNA was extracted. The RNA was fractionated on a sucrose gradient. Panel A: Total count profile of fractionated RNA. The X axis represents gradient fractions and the Y axis, TCA-precipitable cpms. Fractions were pooled and the sizes of the RNAs in each pool verified by denaturing gel electrophoresis. Panel B: An aliquot from each pool was electrophoresed on an agarose gel, transferred to Gene Screen, fluorographed, and exposed to X-ray film. Panel C: Radioactively labelled RNA from each fraction was hybridized to filters containing opposite strands of repeated DNA sequence families in excess and the amount of radioactivity which hybridized to each filter quantitated by scintillation counting. (1) and (2) correspond to complementary strands of the different repeated sequence families cloned into M13.

separation was chosen to separate RNA polymerase II and III Alu products since it is not possible to pulse label newly synthesized RNA in the presence of the RNA polymerase inhibitor, α-amanitin, due to its poor uptake by intact cells. Sucrose gradient centrifugation separated the snRNAs which are the major RNA polymerase III-dependent transcripts from the larger, polymerase II-dependent hnRNAs.

Mouse hepatoma cells were pulse labelled with ^{3}H-uridine and the nuclear RNA was isolated as described in the Materials and Methods. Figure 1A shows the total incorporated count profile and indicates the gradient pools used in hybridization. The direction of centrifugation was from right to left. The pooled RNA fractions which were used for hybridization to cloned repeated DNA sequences were analyzed by denaturing gel electrophoresis to ensure that aggregation had not occurred (Figure 1B). snRNA was only found in the smallest size pool, fraction IV, and did not contaminate the hnRNA fractions (I-III). The pooled RNA fractions were hybridized to each strand of a cloned mouse Alu DNA sequence and 3 non-Alu repeated sequences immobilized on filters (Figure 1C). The amount of transcription homologous to either strand in each RNA fraction was quantitated by scintillation counting of the hybridized transcripts. This experiment was done twice; quantitation from each experiment is shown in Table 1. Fraction IV, containing the RNA polymerase III snRNA products, accounted for 11-27% of the total Alu homologous transcription. The majority of transcripts in this fraction hybridized to Alu strand 1 rather than strand 2 (3-6 fold difference). This was expected since strand 1 in this study corresponds to the template strand for RNA polymerase III. Studies have demonstrated that the Alu family promoters are organized in a bipartite structure similar to the tRNA split promoters (23). Most Alu RNA polymerase III transcription in the cell gives rise to a discrete species, the 4.5S RNA (22,24). Fraction IV Alu transcripts would be predominantly this RNA.

Most Alu-homologous nuclear transcripts (73-89%) were in the hnRNA fractions (I-III). In contrast to the snRNA, the majority of large Alu-related sequences hybridized to strand 2 rather than strand 1. Many of these Alu sequences are presumed to be in intervening sequences and untranslated regions of structural genes (5,26). If Alu members in intervening sequences of structural genes have no function they should be randomly positioned in genes, and both strands should hybridize an equal number of counts. Our analyses indicate that Alu sequences are non-randomly oriented relative to long transcription units. It is interesting that the strand represented more

TABLE 1
Strand Representation of RNA polymerase II and III Alu Family Transcripts In Vivo

		strand #1	strand #2	strand 1 ratio: strand 2
Experiment #1	hnRNA	812[a]	2309	.35
	snRNA	378	127	2.97
Experiment #2	hnRNA	584	1966	.29
	snRNA	1203	202	5.95
Control[b]		5724	4774	.83

[a] total ^{3}H-uridine counts which hybridized to the single-stranded Alu DNA clone.
Input counts for hnRNA, experiment #1 = 1.65×10^{7}; experiment #2 = 1.13×10^{6}
Input counts for snRNA, experiment #1 = 1.67×10^{7}; experiment #2 = 1.8×10^{6}

[b] Nick translated mp8 was hybridized to duplicate filters onto which complementary strands of Alu repeats had been bound. Resulting X-ray films of mp8 hybridization were scanned. These numbers represent the integral of the area under the peaks and are the average of 5 scans.
Mouse hepatoma cells were pulse labelled with ^{3}H-Uridine as described in Materials and Methods. The newly synthesized transcripts were separated on a sucrose gradient into snRNA (fraction IV, Figure 1) and hnRNA (fractions I-III, Figure 1) pools which were hybridized to clones of the complementary strands of the Alu family in excess on nitrocellulose filters.

frequently in long RNA polymerase II transcripts is complementary to the strand of the Alu polymerase III transcripts such as the 4.5S RNA which associates with hnRNA (24).

Although unlikely, the possibility remained that RNA polymerase III transcripts were present in gradient pools I-III. Any RNA polymerase III transcripts found in Fractions I-III would have to read for several kilobases through other Alu family members, most of which would have to be oriented in the opposite direction relative to RNA polymerase III expression in order to give the strand asymmetry seen in Figure 1C. Previous *in vitro* studies of Alu polymerase III transcription demonstrated that most products are less than 600 nucleotides long (22,23). One study did show an exceptionally long *in vitro* product that extended 2000 nucleotides (34). We carried out elongation of transcripts in isolated nuclei in the presence and absence of α-amanitin concentrations that would inhibit RNA polymerase II (0.6 μg/ml) and RNA polymerase III (150 μg/ml). These RNAs were then fractionated by sucrose gradient centrifugation and hybridized as above to strands 1 and 2 of the

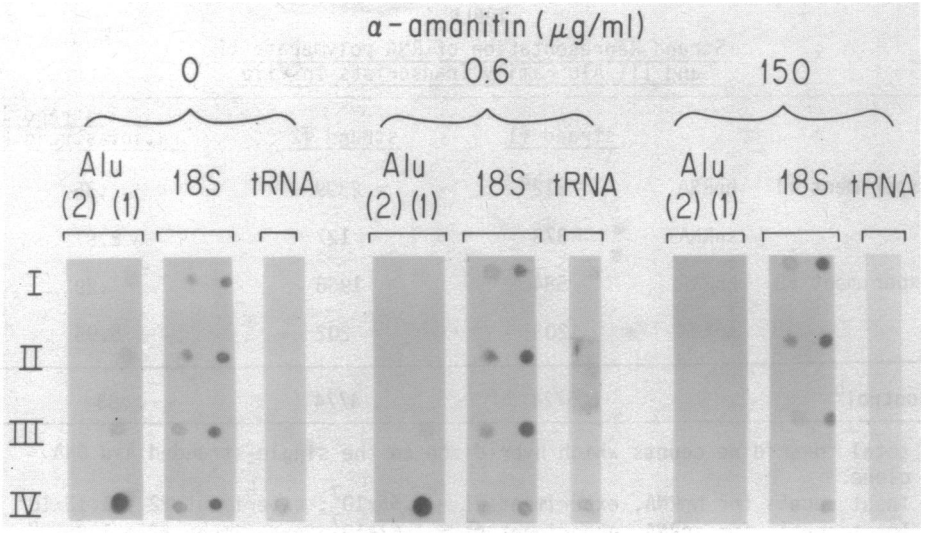

Figure 2: Size Distribution of RNA Polymerase III Alu Transcripts.

α-^{32}P-UTP labelled nuclear RNA was prepared from mouse hepatoma cells by isolated nuclear elongation assays in the presence of 0, 0.6 μg/ml or 150 μg/ml α-amanitin as described (5). RNA was size fractionated as in Figure 1 and hybridized to complementary strands of the Alu family (1) and (2). 18S represents 1 and 5 μg of denatured 18S rDNA as a standard for RNA polymerase I which is unaffected by these concentrations of α-amanitin. tRNA represents 1 μg of a DNA clone for tRNA met as a control for RNA polymerase III.

mouse Alu clone. Figure 2 shows the result of such an experiment. There is very little α-amanitin resistant material (at 0.6μg/ml) in Fractions I-III. The vast majority of RNA polymerase III Alu transcripts resides in Fraction IV and is complementary to strand 1. Overrepresentation of polymerase III products relative to polymerase II is a general feature of isolated nuclear assays. This is due to reinitiation by polymerase III in the absence of polymerase II initiation. These results confirm the proposal that the polymerase III Alu transcription products are predominantly snRNAs, and not long transcripts associated with the hnRNA pool.

Strand Representation of Non-Alu Family Repeated DNA Sequences in Newly Synthesized Nuclear RNA.

We previously reported that the non-Alu repeated sequence families LLRep1, Bam5 and R have RNA polymerase II-dependent transcripts in mouse tissues and cultured cells (5). The Bam5 and R repeats correspond to about

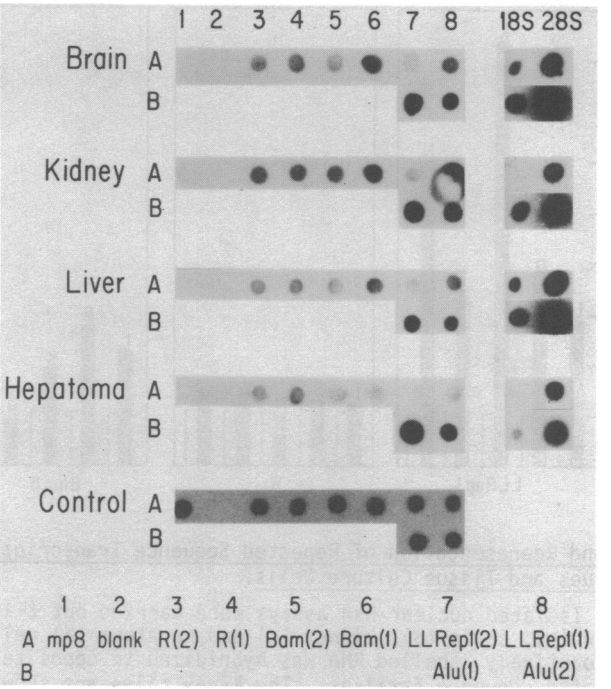

Figure 3: Strand Symmetry of Repeated Sequence Transcription in Isolated Nuclei.

α-^{32}P-UTP-labelled nuclear RNA prepared from indicated sources was hybridized to same DNAs used in Figure 1. DNA was spotted onto nitrocellulose filters which were exposed to X-ray film following hybridization. Each hybridization contained 10^6 cpm/ml. A key to the identity of the DNA samples is shown at the bottom of the Figure. The control panel represents hybridization of nick translated M13RF DNA to an identical filter to ensure equal quantities of DNA were loaded onto each spot. 18S and 28S represent ribosomal DNA clones included as controls for transcriptional activity.

0.5 kb each of the 3' end of the long MIF-I family of repeats and are present in approximately 20,000 copies in the genome (6). The LLRepl family is about 1000 bases long and is represented by 14,000 copies in the genome (5). To assess whether transcripts homologous to these three families are the product of read-through from structural gene promoters or whether the repeated sequences possess their own promoters, both the size distribution and strand representation in transcripts of these non-Alu repeated sequences was examined. The measurements of strand representation were made in 3 mouse

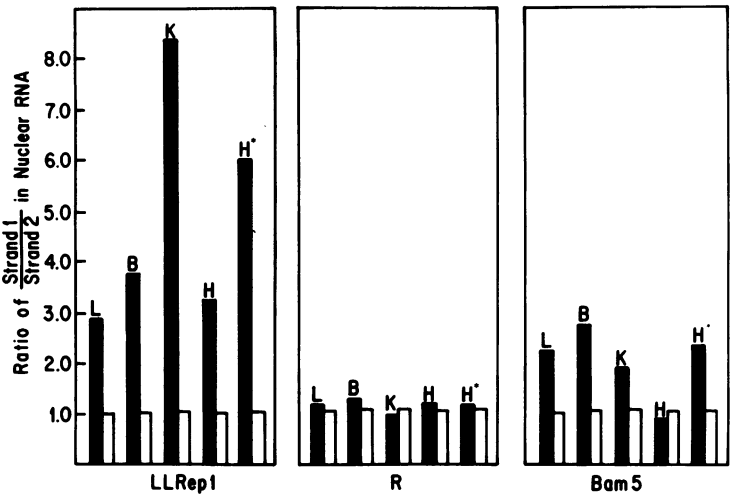

Figure 4: Strand Representation of Repeated Sequence Transcripts in Mouse Tissues and Tissue Culture Cells.

Isolated nuclear RNA assays were carried out (Figure 3) on liver (L), brain (B), kidney (K) and hepatoma (H) cells. Radioactively labelled RNA was hybridized to opposite strands of repeated sequence families. The X-ray films are shown in Figure 3. The films were scanned by densitometry and the areas under the peak integrated. H* represents hepatoma cells pulse-labelled *in vivo* with ^{3}H uridine and analyzed by quantitative filter hybridization (Figure 1B). Histograms represent the ratio of hybridization obtained to strand 1 (closed bars) relative to strand 2 (open bars) with strand 2 set abritrarily constant at 1.0.

tissues as well as the cultured hepatoma cell line. The analyses in mouse tissues used RNA elongated in isolated nuclei since it is not possible to effectively pulse label RNA in intact tissues (Figures 3,4). The size distribution of these non-Alu repeated sequence transcripts was determined in hepatoma cell newly synthesized RNA as described above (Figure 1).

Table 3 summarizes the hybridization of both the *in vitro* (isolated nuclei) and *in vivo* (pulse labelled) RNAs to each strand of the LLRep1, Bam5 and R family DNAs. The LLRep1 family is asymmetrically transcribed at levels from 2.7 to 8.6-fold. Bam5 shows some asymmetry up to 3-fold but it is not as marked as that seen for LLRep1. The R family, which is frequently linked to Bam5 as part of the MIF-1 family in the genome, does not show asymmetric strand representation in transcripts. While the strand ratio for each family varies quantitatively from one tissue to another, the direction of asymmetry for LLRep1 and Bam5 remains the same amongst the tissues examined, using two

Table 2

Hybridization of Pulse-Labelled Nuclear RNA to Complementary Strands of Non-Alu Repeated DNA Families

		Fraction				
Family		I	II	III	IV	
LLRep 1	Expt. #1	930	183	222	178	Strand 1
		112	38	29	42	Strand 2
	Expt. #2	570	242	42	58	Strand 1
		135	70	35	20	Strand 2
Bam5	Expt. #1	1385	247	93	97	Strand 1
		312	136	85	51	Strand 2
	Expt. #2	544	190	56	61	Strand 1
		351	276	38	37	Strand 2
R	Expt. #1	410	107	101	69	Strand 1
		274	113	90	63	Strand 2
	Expt. #2	467	196	45	39	Strand 1
		334	264	32	42	Strand 2
Input Counts	Expt. #1	6×10^6	1.2×10^7	3.5×10^6	1.8×10^6	
	Expt. #2	1.1×10^7	3.5×10^6	2.3×10^6	1.1×10^6	

Pulse-labelled RNA was prepared as in Table I and was hybridized to the complementary strands of the LLRep1, Bam5 and R families.

[a] Numbers represent cpm hybridized.

different labelling methods. Examination of size fractionated RNA (Figure 1C) shows that all 3 non-Alu families hybridized to nuclear RNA whose size distribution is that of hnRNA. No predominance of discrete length transcripts was apparent.

Synthesis of Repeated Sequence Transcripts in Tissues and Cultured Cell Lines

One hypothesis concerning the function of repeated sequences is that their transcripts are involved in the regulation of gene expression. We wanted to determine whether repeated sequence transcription varied quantitatively in different tissues and cultured cells. To address this issue, assays of elongation in isolated nuclei were carried out using mouse brain, liver, kidney or cultured hepatoma cell nuclei as described above. The

Table 3

Strand Ratios of Non-Alu Repeated DNA Sequences in Nuclear RNAs

			In vitro[b]			
Family	In vivo (#1)[a]	In vivo (#2)[a]	B	L	K	Hep
LLRep1	3.5	6.9	3.64	2.68	8.6	3.21
Bam5	1.06	3.11	2.6	2.25	1.7	.9
R	.88	1.52	1.2	1.1	1.1	1.05

[a] Two experiments were carried out as described in Materials and Methods by labelling mouse hepatoma cells for 10 minutes with ^{3}H-uridine. Strand ratios were derived from a total of the cpm hybridized in fractions I-IV.

[b] Nuclei were isolated from the indicated sources and elongating RNA was labelled with (α-^{32}P) UTP for 15 minutes. B = brain, L = liver, K = kidney, Hep = hepatoma.

This table summarizes the hybridization of nuclear transcripts to the non-Alu repeated seqence families LLRep1, Bam5 and R (figures 1,2,3; Table 2). Labelled transcripts were prepared as described in Materials and Methods by either pulse-labelling (in vitro).

total transcriptional activity varied from tissue to tissue as demonstrated by variation in the hybridization of labelled transcripts to 18S and 28S rDNA clones. While repeated sequence transcripts varied quantitatively between tissues and cultured cells, no consistent pattern could be observed when the repeated sequence counts were normalized to ribosomal RNA synthesis. Thus, the Alu, Bam5, R and LLRep1 families do not appear to be differentially regulated amongst the tissues examined.

DISCUSSION

In this study, we have determined that the majority of Alu-homologous transcription in the cell is due to read-through from adjacent structural genes since Alu sequences themselves do not have RNA polymerase II promoters. Alu sequences have been found in the 3' untranslated regions of mRNAs and in the intervening sequences of several genes (see 5). We have shown that the orientation of Alu sequences in these transcription units is non-random. The rodent Alu family also gives rise to discrete RNA polymerase III transcripts predominated by the 4.5S RNA (24). The orientation of Alu sequences in structural genes occurs preferentially so that the strand most often transcribed by polymerase II is complementary to that of the 4.5S RNA. It was previously reported that the 4.5S RNA was reversibly associated with hnRNA and mRNA (24) leading to a hypothesis that the 4.5S RNA is involved in

RNA processing (33). Our results are consistent with this view.

The transcriptional measurements presented here are more consistent with the hypothesis that RNA polymerase II transcripts of most repeated sequence families, including Alu, arise by read-through from nearby genes rather than as a result of specific transcription of the repetitive elements themselves. This conclusion arises from the finding that newly labelled RNA hybridizes to both strands of the Alu, Bam5 and R families and that these transcripts are heterogeneous in size. The LLRep1 family exhibits a more asymmetric pattern of hybridization of nuclear RNA than Bam5 and R. However, while Bam5 and R repeats have been found in intervening sequences of at least 2 structural genes, LLRep1 has not been seen in any of 10 genes analyzed (LL, unpublished and 5). These facts suggest that members of the LLRep1 family may have their own promoters, or that they are generally oriented in only one direction relative to trancription units. Either case is intriguing. The transcriptional patterns of the Bam5 and R families are distinct from each other in that Bam5 is transcribed somewhat asymmetrically while both strands of the R family sequence are equally transcribed. This finding is interesting in light of the genomic structure and organization of these two repeated sequence families. Bam5 and R are frequently juxtaposed in the genome and as such, form the 3' end of the long repeated sequence family MIF-1 (6,7). These findings suggest that some Bam5 family members may be transcribed from their own promoters or be preferentially oriented in certain transcription units. Future experiments will be directed at studying transcription of different individual members of the LLRep1 and Bam families in a cell-free transcription system.

The total expression of these 4 repeated sequence families does not appear to be differentially regulated in the 3 mouse tissues and the cultured cell line examined in this study. This is in marked contrast to our observations in steady state RNA populations in which repeated sequences accumulate in cultured cells to a much higher extent than in normal mouse tissues (Heller et al., in preparation). In addition, we have found that the strandedness of newly synthesized transcripts does not vary from tissue to tissue. Since these studies have dealt with all transcripts homologous to a repeated sequence family, they do not eliminate the possibility that transcription from particular family members may be developmentally regulated as has been observed for the sea urchin repeated sequence families (16).

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