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Human Ribosomal DNA Fragments Amplified in Hamster Cells Are Transcribed Only by RNA Polymerase II and Are Not Silver Stained

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Cloned human rRNA gene fragments that included the promoter region were introduced into Chinese hamster dihydrofolate reductase-deficient (dhfr$^-$) cells by cotransformation with a *dhfr* minigene and amplified by selection for methotrexate resistance. The human ribosomal DNA was transcribed by RNA polymerase II, not RNA polymerase I or III. The metaphase chromosome regions containing the transcriptionally active human ribosomal DNA failed to show silver staining.

A surprising feature of the human rRNA genes, which are transcribed by RNA polymerase I, is that under artificial conditions, the promoter region can initiate transcription that is mediated by RNA polymerase II, especially if upstream ribosomal DNA (rDNA) promoter sequences from −650 to −83 are deleted (19). Transcription by either polymerase requires the formation of a stable complex between the promoter and protein cofactors (4, 6). Polymerase I-mediated transcription is modulated by the coordinate binding of two factors to an upstream promoter element at −156 to −107 (12). The fact that transcription of rRNA is normally mediated only by RNA polymerase I may indicate that polymerase I cofactor binding to this upstream control element blocks polymerase II cofactor binding to the promoter. If so, the absence of polymerase I cofactors should favor polymerase II-mediated transcription of rDNA even in the presence of the upstream control region.

To test this idea, we introduced human rRNA gene fragments containing the promoter, including sequences up to −500 nucleotides from the start site, into Chinese hamster cells. Human RNA polymerase I transcription cofactors were not present in this system, which might then favor RNA polymerase II-mediated transcription of the rDNA fragments.

Chinese hamster dhfr$^-$ cells (20) were cotransfected by the calcium phosphate precipitation technique (23) with a mixture containing 0.1 μg of pMg3 DNA with a mouse *dhfr* minigene (7) and 5 μg of linearized pBEs DNA containing a 1.2-kilobase (kb) segment of human rDNA (24) (Fig. 1). dhfr$^+$ transformants were isolated and designated HUR. Southern blot analysis of HUR DNAs digested with *Kpn*I and hybridized (22) to the nick-translated 1.2-kb fragment of human rDNA from pBEs showed that human rDNA sequences were present, mostly as high-molecular-weight DNA, in 75% (18/24) of the transformants analyzed. The human rDNA fragment used as the probe did not cross-hybridize to the endogenous Chinese hamster rDNA (Fig. 2). Transformed cells resistant to 5×10^{-6} M methotrexate were obtained by stepwise increases in drug concentration (1) and were found to have coamplified both the *dhfr* minigene and the human rDNA (Fig. 3). Southern blots of *Eco*RI-*Sal*I-digested DNA from all the resistant clones showed, in addition to the human rDNA fragments seen in the original transformant DNA, prominent extra bands, indicating that some rearrangements had occurred during amplification of the DNA. Independently derived methotrexate-resistant

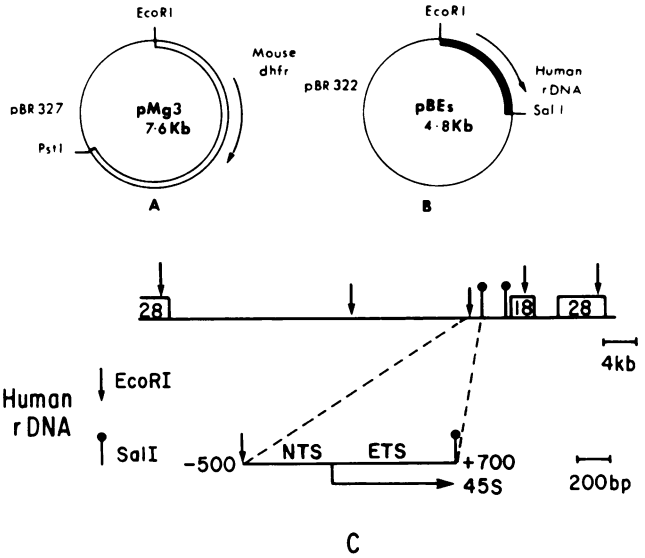

FIG. 1. Recombinant plasmids used in the study were pMg3 containing a 5.1-kb mouse *dhfr* minigene inserted into pBR327 (7) (A) and pBEs containing a 1.2-kb *Eco*RI-*Sal*I fragment of human rDNA that includes some nontranscribed spacer (NTS), the promoter, and external transcribed spacer (ETS; 24) (B and C).

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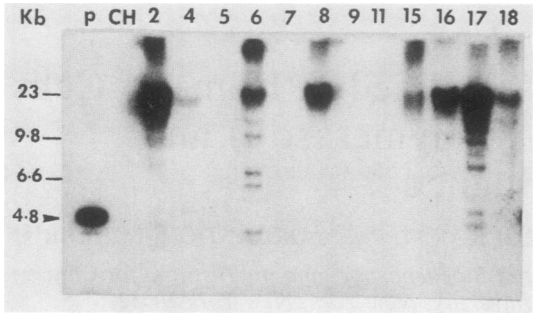

FIG. 2. Identification of human rDNA sequences in Chinese hamster dhfr$^+$ (HUR) cell lines. Lanes: p, 70 pg of DNA from linearized plasmid pBEs; CH, 10 μg of parental Chinese hamster dhfr$^-$ cells; 2 to 18, 10 μg each of cell clones HUR-2 to HUR-18. Southern blots of *Kpn*I fragments were hybridized to the ^{32}P-labeled 1.2-kb insert from pBEs (specific activity, 4×10^7 cpm/μg).

subclones had different patterns of amplification of the human rDNA (data not shown).

Transcription of human rDNA was shown by using dot blots of purified total cellular RNA (3, 8) from the HUR transformants. A low level of hybridization was visible with RNA from the original transformants, and a much higher level was visible with RNA from cells with amplified human rDNA sequences (Fig. 4). No hybridization was detected with RNA from Chinese hamster dhfr$^-$ cells. Rates of transcription were determined by nuclear run-on assays (13). Isolated nuclei (9) were allowed to transcribe in reactions containing [^{32}P]UTP with or without actinomycin D or α-amanitin for 30 min at 25°C. ^{32}P incorporated into RNA was counted after trichloroacetic acid precipitation of duplicate samples. The amount of [^{32}P]RNA that hybridized to filters containing 0.5 μg of single-stranded DNA from either the 1.2-kb human rDNA probe or calf thymus was determined from scintillation counts. The transcription rate (in parts per million), calculated as [(counts per minute bound to human rDNA − counts per minute bound to calf DNA)/input counts per minute × 10^6], was three- to sixfold higher in methotrexate-resistant subclones than in HeLa cells (Fig. 5 and 6). Thus, the transferred human rDNA was expressed, and the steady-state level of transcription increased to high levels after an increase in the number of gene copies. By comparisons with HeLa cells, it appears that the steady-state level of transcripts was lower than that expected from

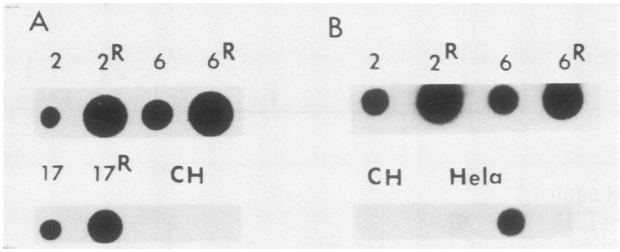

FIG. 3. Amplification of mouse *dhfr* and human rDNA sequences in methotrexate-resistant Chinese hamster clones shown by DNA dot-blot hybridization. Total cellular DNA (2 μg) from original HUR clones (HUR-2, HUR-6, and HUR-17), methotrexate-resistant (5×10^{-6} M) subclones (HUR-2^R, HUR-6^R, and HUR-17^R), and Chinese hamster dhfr$^-$ cells (CH) was spotted and hybridized with ^{32}P-labeled mouse *dhfr* insert (specific activity, 4×10^7 cpm/μg) (A) or ^{32}P-labeled 1.2-kb human rDNA insert (specific activity, 3×10^7 cpm/μg) (B). HeLa cell DNA (0.2 or 0.5 μg) was used as a control.

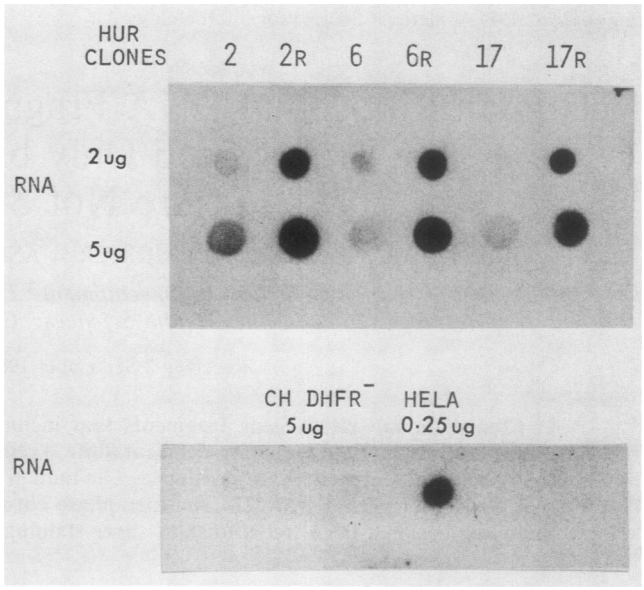

FIG. 4. Expression of human rDNA sequences by transformed Chinese hamster cells. The ^{32}P-labeled 1.2-kb rDNA insert (specific activity, 2×10^7 cpm/μg) was hybridized to dot blots of total cellular RNA from HUR-2, HUR-6, and HUR-17; their methotrexate-resistant derivatives, HUR-2^R, HUR-6^R, and HUR-17^R; parental hamster cells (CH); and HeLa cells.

the rate of transcription, perhaps reflecting their more rapid degradation in a nonnucleolar location.

To determine which polymerase(s) mediated this transcription, drug inhibition studies were carried out. Low concentrations of actinomycin D inhibit RNA polymerase I-mediated transcription from rDNA but have little effect on RNA polymerase II-mediated transcription (17). Nuclear run-on assays of transcription in the presence or absence of 0.08 μg of actinomycin D per ml showed that the drug had no effect on the transcription of either human rDNA or mouse *dhfr* in HUR-6^R nuclei but completely blocked human rRNA synthesis in HeLa nuclei (Fig. 5). The failure of actinomycin D to abolish human rDNA transcription in the transformed

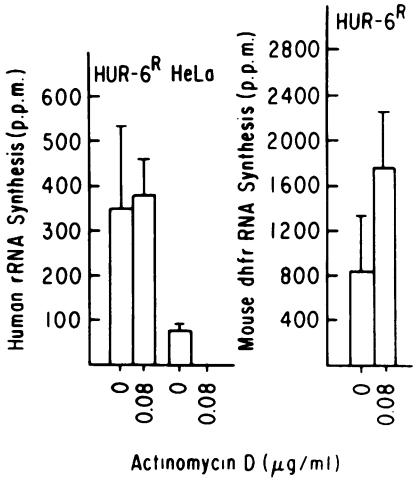

FIG. 5. Effect of actinomycin D on human rDNA and mouse *dhfr* synthesis in HUR-6^R-transformed hamster cells. Nuclear run-on assay was described in the text.

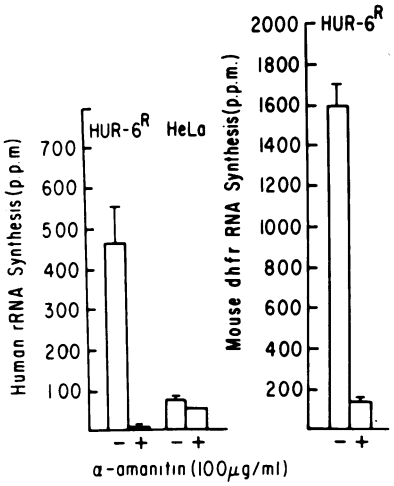

FIG. 6. Effect of α-amanitin on synthesis of human rRNA or mouse *dhfr* RNA in HUR-6^R-transformed hamster cells. Nuclear run-on assay was as described in the text.

hamster cells indicates that this rDNA was not transcribed by RNA polymerase I. This was confirmed in experiments with HUR-6^R and HeLa nuclei exposed to 100 μg of α-amanitin per ml, which inhibits transcription by RNA polymerases II and III but not by polymerase I (2). α-Amanitin blocked 94 to 98% of the transcription of both human rDNA and mouse *dhfr* sequences in the transformed cells but did not inhibit rRNA synthesis in HeLa cells (Fig. 6). These results indicate that in transfected hamster cells, unlike in HeLa cells, the human rDNA was not transcribed by RNA polymerase I.

To determine whether the rDNA transcription was mediated by RNA polymerase II or III, the nuclear run-on experiment was repeated with nuclei from a similar transformed line and α-amanitin at a concentration of 1 μg/ml, which inhibits only RNA polymerase II (2). This change abolished the transcription of both human rDNA and mouse *dhfr* but had no effect on the polymerase III-mediated transcription of tRNA^{Arg} (Table 1). We conclude that transcription of the transfected human rDNA was mediated by RNA polymerase II alone.

rRNA gene clusters that have been transcriptionally active can usually be detected by silver staining of metaphase chromosomes (15). However, in three independent methotrexate-resistant hamster clones (HUR-6^R, HUR-20^R, and HUR-31^R), the amplified human rDNA sites failed to show silver staining, although the endogenous nucleolus organizer regions at the ends of several hamster chromosomes did (Fig. 7). We conclude that silver staining is restricted to rDNA chromatin that is competent for polymerase I-mediated transcription.

TABLE 1. Nuclear run-on assay of α-amanitin inhibition of RNA synthesis

Sample	Amt of [^{32}P]RNA bound to blots[a]	
	Without α-amanitin	With α-amanitin (1 μg/ml)
Human 1.2-kb rDNA fragment	111.1 ± 5.2	2.5 ± 0.5
Mouse 3.4-kb *dhfr* minigene	167.9 ± 2.7	6.0 ± 0.3
tRNA^{Arg} DNA	157.1 ± 2.2	129.0 ± 1.7

[a] In arbitrary units, estimated by densitometry of autoradiographs.

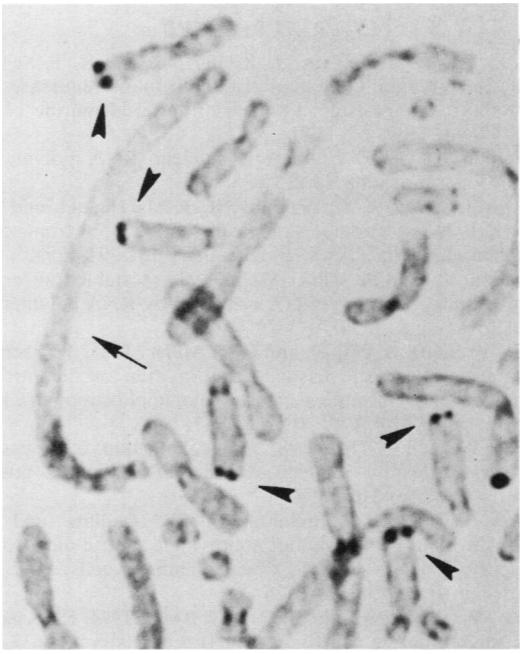

FIG. 7. Metaphase cell from HUR-31^R stained with silver nitrate to show nucleolus organizer regions. Arrowheads indicate Chinese hamster nucleolus organizer regions. Arrow indicates an amplified region which lacks silver staining.

Polymerase I specificity is conferred on the human rDNA promoter by the coordinate binding of two factors, one of them species specific (11), to an upstream control region (12); thus, polymerase I-mediated transcription is abolished, and abundant polymerase II-mediated transcription of the rDNA ensues in gene transfer systems in which this upstream control region is deleted (19) or the human-specific factor is absent (this report). It is unclear, in the present cotransformation study, whether the polymerase II-mediated rDNA transcription used the rDNA promoter or the *dhfr* promoter, but in the transient expression system used by Smale and Tjian (19), it used the rDNA promoter. Mouse-human hybrid cells are also marked by the absence of human polymerase I transcription cofactors (10, 14, 16), but even when they contain many copies of the human rRNA genes, they show no transcription of human rDNA (15, 18). Why is the human rDNA in the hybrid cells not transcribed by polymerase II? One explanation is that nontranscribed spacer sequences, which are present in the hybrid cells but not in the transfectants, bind proteins that influence promoter specificity or its availability for cofactor binding. Such an explanation could account for the low level of transcription of truncated mouse rDNA fragments introduced into hamster (5) and rat (21) cells and the 10-fold enhancement of polymerase I-mediated rDNA transcription that is produced by the inclusion of 8 kb of nontranscribed spacer in the transfected rDNA (21).

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