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The Mouse Ribosomal DNA Promoter Has More Stringent Requirements In Vivo than In Vitro

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Using mouse ribosomal DNA templates bearing polymerase I terminators to prevent transcriptional interference (S. L. Henderson, K. Ryan, and B. Sollner-Webb, Genes Dev. 3:212–223, 1989) and facilitate promoter analysis in intact cells, we demonstrate that a −140 promoter domain (as well as the core region) is essential for appreciable levels of initiation in vivo. This in vivo polymerase I promoter can also be detected in vitro but only under very stringent conditions.

Transcription of eucaryotic rRNA genes in vitro has been studied extensively, showing principally a core promoter domain (residue ~−35 to ~+5) (8, 11, 14,16–19, 25, 30, 31, 33, 37–39; reviewed in reference 32). Essential transcription factors bind the upstream half of this region, allowing binding of the active polymerase; initiation requires the entire core promoter (3, 14, 18, 32, 34, 35). However, two in vitro studies utilizing stringent reaction conditions showed substantial stimulatory effects of sequences extending upstream to residue ~−140 (11, 25).

When mouse 5′ deletion mutants were analyzed in transiently transfected rodent cells, significant levels of initiation required sequences surrounding residue −168 (12). However, this −168 element is a transcriptional terminator and behaves unlike a promoter element, being position independent and completely replaceable by other termination effectors such as the 3′ end of the pre-rRNA region, pyrimidine dimers, or discontinuities in the template. Without functional terminators, polymerases progress around the circular plasmid and through the promoter, releasing the bound initiation factors and thereby preventing subsequent initiations from that transcription complex (13). Despite these findings and the elucidation of an adjacent upstream enhancer element (29), the mouse ribosomal DNA (rDNA) promoter functioning in intact cells remained to be defined.

To determine the mouse rDNA promoter in vivo, 5′ and 3′ deletion mutants reconstructed to contain polymerase I terminator elements were introduced into rodent cells. The inserted terminator enables deletion mapping of the promoter, unobscured by transcriptional interference that earlier prevented in vivo promoter determination (13).

5′ Border of mouse rRNA gene promoter in transiently transfected cells. For the 5′ deletion series 5′Δ/3′T, polymerase I terminator elements from the 3′ end of the 47S pre-rRNA region (9) were inserted into plasmids bearing 5′ deletions of the mouse rDNA promoter region (12, 25). Transient transfections of these templates were analyzed by S1 mapping (Fig. 1). Templates containing ≥149 rDNA residues upstream of the initiation site were efficiently transcribed, whereas templates bearing more-extensive 5′ deletions exhibited minimal transcription. 5′Δ−126 transcribes ~2 to 5% as efficiently as larger templates (Fig. 1), and 5′Δ−41 can also direct perceptible levels of expression at elevated amounts of input template (Fig. 1B and data not shown). 5′Δ−35 and more-extensive deletions direct no detectable transcription, even in considerably overexposed autoradiograms.

Thus a promoter domain extending upstream to residue ~−140 is virtually essential for rDNA transcription in transfected cells. In vitro, this domain stimulates transcription under stringent but not under the usual relaxed conditions (25). The core promoter domain (5′ border at residue ~−41), which directs efficient expression under most in vitro transcription conditions (25), directs only minimal transcription in vivo (Fig. 1B).

These results extend earlier transfection studies showing initiation, but of an indeterminate level, by the mouse core promoter domain. In one set of experiments (12), templates lacking both the terminator and upstream promoter domains yielded only small amounts of RNA extending many times around the plasmid, but transcription complex disruption by the traversing polymerases could have reduced the apparent initiation level. Transcription reported from a mouse 5′Δ−39 mutant in transfected cells (10) was also of an uncertain level because the reverse transcriptase yielded mainly incomplete extension products. In human rDNA, although read-in transcriptional interference may affect interpretation of deletion analysis (1, 31), a study of internal mutations showed that a ~−140 domain is very important in transfected cells (15), a finding consistent with the findings for mice. rDNA promoters have also been examined using intact cells of two nonmammalian species. In *Xenopus* oocytes, abundant transcription was initially observed without the −140 region (33), but at lower template concentrations, the −140 domain is necessary (37). The yeast rDNA promoter also utilizes sequences around residue ~−140 (26). Thus, a promoter domain at residue ~−140 is required for active in vivo rDNA transcription in widely divergent organisms, even though their primary sequences are not conserved.

Figure 1 also demonstrates that mouse rDNA sequences between −147 and −230 (where the rDNA enhancer begins; 29) do not confer stimulation beyond preventing transcriptional interference. This is unlike the situation in *Xenopus*

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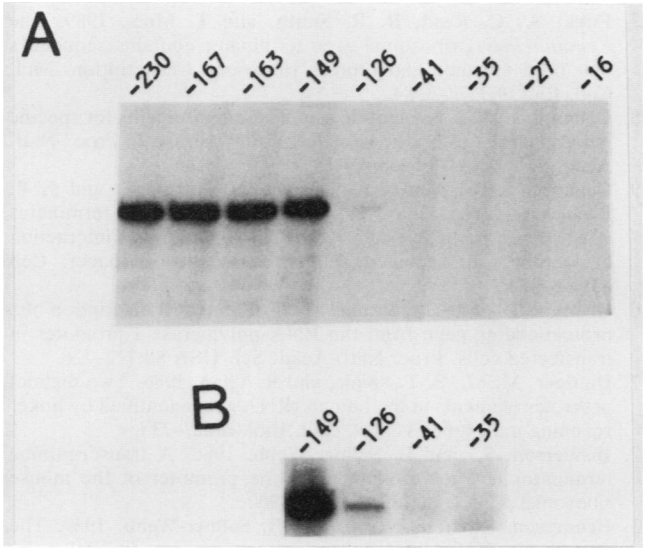

FIG. 1. 5′ Border of mouse rDNA promoter assayed in transfected cells. The 5′Δ/3′T series of deletion mutants was transfected into CHO cells by using a 4-h DEAE-dextran treatment (200 μg/ml) and a dimethyl sulfoxide shock as described elsewhere (12, 21). The DNA concentration in the transfection solution was the usual 2 μg/ml (A) or was elevated to 32 μg/ml (B). The 5′Δ/3′T templates were constructed from the original 5′ deletions (12, 25) by insertion of a 1.9-kilobase *Sma*I-*Nde*I fragment (beginning 55 nucleotides downstream of the 28S coding region and containing the eight tandem rDNA 3′ terminators) between rDNA residue +292 and the *Nde*I site of the pBR322 vector. RNA was isolated 24 h posttransfection (2, 21), and 5 μg was analyzed by S1 nuclease mapping with a 5′-end-labeled mouse-specific probe and denaturing 4% polyacrylamide gels (12). The position of the most 5′ rDNA residue of each mutant is shown above the lane.

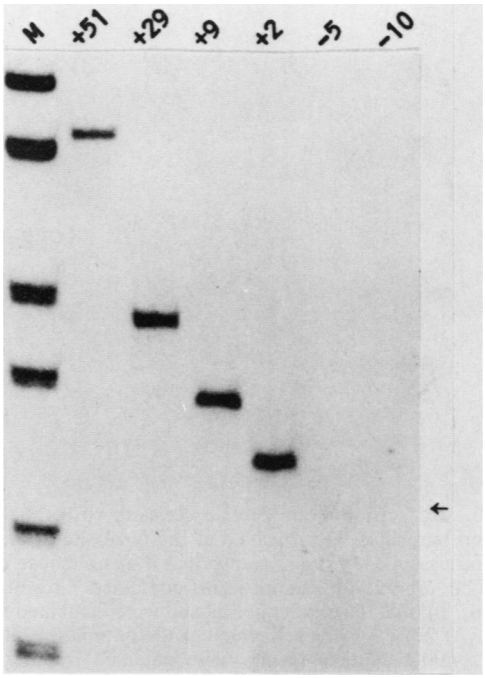

FIG. 2. 3′ Border of mouse rDNA promoter in transfected cells. The −230/3′Δ series (reconstructed from deletions extending to residue −126 [25] to contain mouse rDNA between residue −230 [*Eco*RI linker] and the indicated 3′ position [*Bam*HI linker] and cloned in pBR322) was transfected into CHO cells as for Fig. 1A. RNA (10 μg) was analyzed by primer extension (22) using avian myeloblastosis virus reverse transcriptase (Life Sciences) and a 38-nucleotide primer, residues 434 to 471 of the transcript (*Nar*I to *Ban*II of pBR322). Extension products were resolved on a denaturing 4% polyacrylamide gel. Lane M shows the pBR322 *Hpa*II marker bands between 160 and 76 nucleotides. To prepare the labeled primer, *Nar*I-cleaved pBR322 (2 pmol) was treated with T4 DNA polymerase (1.25 U/μg of DNA; Bethesda Research Laboratories) for 1.5 min at 37°C in the absence of nucleotides to allow 3′ to 5′ exonuclease activity; then dTTP, dGTP, [α-^{32}P]dATP, and [α-^{32}P]dCTP were added for 60 min, and the primer (1×10^6 to 3×10^6 dpm/pmol) was released by *Ban*II digestion and isolated on an 8% polyacrylamide strand separating gel.

laevis, in which transcriptional stimulatory sequences reside in or overlap the promoter-proximal terminator (7, 23).

3′Border of mouse rDNA promoter. Similar transfection analyses were performed to determine the 3′ extent of the promoter in vivo. 3′ Deletions were reconstructed to contain rDNA through position −230, including the −168 promoter-proximal terminator. Following transfection into rodent cells, RNA from this −230/3′Δ series was analyzed by primer extension. The rDNA promoter extends 3′ to the initiation site (Fig. 2); 3′Δ+2 directs active transcription, as do the larger templates, while 3′Δ−5 and more-deleted templates are virtually inactive. A very faint signal (indicated by the arrow in Fig. 2) could be detected from 3′Δ−5; 3′Δ−10 yielded no analogous transcript.

Earlier in vitro assessment of −126/3′Δ deletions showed an apparent 3′ promoter border between +2 and +9 (25), in apparent contrast to the current observations (Fig. 2). This difference was not due to different requirements in vivo versus in vitro or to sequences between −230 and −127 substituting for information between +2 and +9 but to different in vitro conditions exhibiting different 3′ requirements (Fig. 3). When the −230/3′Δ series (Fig. 3A) and the original −126/3′Δ series (not shown) were transcribed in vitro, both series showed the same 3′ border as in vivo, with 3′Δ+2 fully active, 3′Δ−5 minimally active, and 3′Δ−10 showing no analagous transcript, even at long exposures (Fig. 3A, last three lanes). However, in vitro transcriptions at elevated $MgCl_2$ show markedly reduced transcription of 3′Δ+2 relative to 3′Δ+9 (Fig. 3B), creating an apparent border between +9 and +2. Thus, the previously observed +2/+9 border presumably reflected an elevated effective divalent-ion concentration (25). Since 3′Δ+2 (Fig. 3) and a 3′Δ−1 (27, 39) transcribe efficiently in vitro, while 3′Δ−5 (Fig. 3; 25) anda −1 point mutation (4) do not, the 3′ promoter border isat the initiation site, residue +1, which is consistent with the in vivo determination (Fig. 2). We have never detected effects of the mammalian conserved residues +2 and +18 (6).

In summary, transcriptional initiation of mouse rDNA in transfected cells requires, in addition to a terminator to prevent transcriptional interference (13), an upstream (−140) promoter domain and a core promoter domain. The core domain is essential for transcription in vitro, but the upstream domain has minimal or no effect in vitro under all but the most stringent conditions. The requirement for a −140 promoter domain in vivo across widely divergent species provides additional evidence (5, 28, 29, 36) that components

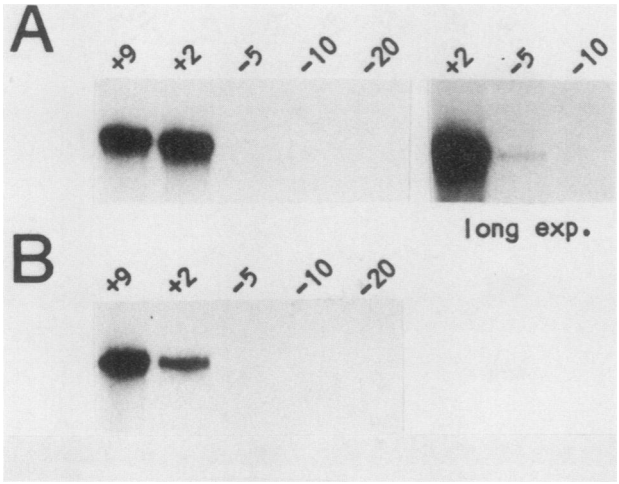

FIG. 3. Transcription of −230/3′Δ series in vitro. The indicated 3′ deletion templates were cleaved at the *Nru*I site in the pBR322 region and assayed by runoff transcription using mouse cell S-100 extract (24). The 25-μl reactions (24) contained 7 μg of template DNA and 7 μl of S-100 extract per ml and were incubated for 45 min at 30°C. (A) Assays were performed at the optimal $MgCl_2$ concentration, 5 mM. Identical results were obtained whether the KCl concentration was 90 or 120 mM, as well as with closed circular templates assayed by primer extension. The last three lanes show a longer exposure. (B) The $MgCl_2$ concentration was increased to 7 mM, causing a diminution in transcription from all templates. (Alterations in KCl concentration did not reproduce this effect.) Unlike the earlier examined 3′Δ+2 (25), which contained an extra eight-nucleotide *Bam*HI linker at the site of the deletion, the current templates were formed with only one linker; the 3′Δ+2 templates exhibited the identical transcription efficiencies and the same initiation site (residue +1) whether they contained one or two linkers (data not shown).

that direct rDNA transcription are functionally and positionally conserved in evolution.

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