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Nucleotide sequence analysis of the spacer regions flanking the rat rRNA transcription unit and identification of repetitive elements

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ABSTRACT

We investigated the organization of the rat rDNA non-transcribed spacer (NTS) by determining the sequence of large NTS segments located upstream (2501 bp) and downstream (4025 bp) from the rRNA transcription unit. We identified four B2-like and two ID mobile elements. They may be grouped in three pairs with the members of each pair located in the upstream and downstream NTS. The ID sequences are identical to the consensus sequence, while the pairs of B2-like elements show 85 % and 50/65 % homology to the consensus B2 sequence. The proximal part of the downstream NTS contains a region of widely diverged SalI tandem repeats. A considerable part of the analyzed upstream and downstream NTS sequences is constituted by different types of simple sequences and long poly(purine)·poly(pyrimidine) tracts. These data show that the rat rDNA NTS regions flanking the rRNA transcription unit are characterized by a combination of short interspersed (B2-superfamily) and various simple sequences.

INTRODUCTION

Sequence analyses of the rDNA repeats in different eukaryotes reveal that while rRNA genes are rather conservative, the non-transcribed spacer (NTS) sequences are widely divergent [1,2]. Despite the rapid evolutionary changes in NTS sequences recent studies provide evidence that they play a definitive regulatory role in the expression of rRNA genes [3]. Elucidation of the complete sequence of NTS in Saccharomyces [4], Drosophila [5,6] and Xenopus [7] and further studies on in vitro and in vivo rDNA transcription showed that duplicated promoter sequences or other NTS elements possess an enhancer-like function [8-12]. It is known that the NTS in Mammalia is markedly larger than that of lower vertebrates, but its structure and possible functions are still largely unknown [2]. Restriction endonuclease and partial sequence analyses revealed the presence in the mouse [13,14], rat [15,16] and human [17] NTS of tandemly repeated sequences analogous to those of lower eukaryotes. In addition, hybridization studies showed the presence in mammalian NTS rDNA of highly repetitive sequences interspersed in the genome [14,15,18-21]. These

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observations suggest the acquisition of additional functions by the oversize mammalian NTS. Their elucidation requires as a first step more detailed and complete sequence analyses.

In the present work we report the sequence of extended NTS segments of the rat rDNA repeating unit located upstream and downstream from the rRNA transcription unit.

MATERIALS AND METHODS

Plasmids. Two recombinant phages, λ RrII and λ RrIV [15], containing the whole rat rRNA transcription unit and about 12 kb of flanking spacer sequences were used to subclone appropriate rDNA fragments (pBR 322 vector). The plasmids pRr 56, pRr 151, pRr 22 and pRr 24 contain different rDNA NTS segments and were used for the sequencing analyses in the present work. Plasmid DNA was isolated and purified by standard procedures [22].

Enzymes. The restriction endonucleases AvaI, DdeI, HinfI and TaqI were products of BioLabs Inc.,Beverly, Mass. U.S.A. BamHI, EcoRI, HindIII, MspI, PstI, SalI and T4 polynucleotide kinase were obtained from NPO "Ferment", Vilnus, USSR; Cfr6I, Cfr13I, Eco47I, Eco52I, HindII, MvaI, Sau3A and XhoI were kindly provided by Dr A.Yanulaitis (Vilnus); EcoRV, KpnI and Klenow fragment of DNA polymerase I by Dr I.Fodor (Puschino,USSR) and SauI by Dr.J. Timko (Bratislava,CSSR). Alkaline phosphatase from E.coli was a product of Worthington, Freehold, New Jersey, U.S.A.

Nucleotide sequence analysis was carried out as described by Maxam and Gilbert [23]. Terminal labeling was made with [γ^{32}P]ATP and T4 polynucleotide kinase or by end filling of protruding restriction endonuclease ends with [α^{32}P]dNTP using the Klenow fragment of DNA polymerase I. [γ^{32}P]ATP was synthesized according to Walseth and Johnson [24] with the use of ^{32}P-inorganic phosphate obtained from Amersham, Bucks, UK or from "Isotope", Tashkent,USSR. [α^{32}P]dNTP were obtained from The Radiochemical Centre, Amersham, Bucks, UK. Direct and reverse homology matrices and comparison with different nucleotide sequences were performed for the computer analysis of nucleotide sequences using a Nova-2 computer.

RESULTS

Sequence of upstream rDNA NTS region. At present, about 7 kb of the rat NTS, located upstream from the transcription start site, have been cloned by three independent groups [15,16,25]. The sequence of about 1.9 kb preceding the transcription start site has been determined, encompassing its immediate

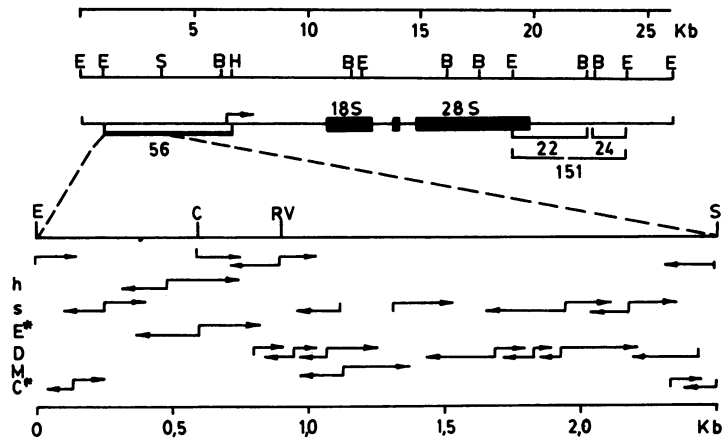

Figure 1. Upper part: General organization and major restriction sites of the cloned rat rDNA segments. Horizontal arrow - transcription start site. The position of the subcloned rDNA fragments in pRr56, pRr22, pRr24 and pRr151 is indicated. The pRr151 includes the pRr22 and pRr24 rDNA plus the intercalated BamHI - BamHI rDNA fragment. For more detailed restriction maps see [15,30].

Lower part: Extended map and sequencing strategy used in the analysis of the EcoRI - SauI subfragment of the rDNA NTS in pRr56. B-BamHI; E-EcoRI; H-HindIII; S-SauI; C-Cfr6I; RV-EcoRV; h-HinfI; s-Sau3A; E*-Eco47I; D-DdeI; M-MspI; C*-Cfr13I. Only the position of the restriction sites generating the DNA subfragments used in sequencing is indicated.

vicinity [26-28] and an upstream MspI tandem repeat region [16]. We have shown previously that the pRr56 rDNA insert used in this work is a variant containing 9 MspI repeats [29]. Partial sequencing of this region (data not shown) revealed the identity of the sequences in our rDNA clone with those published earlier [16]. Here we report the sequence of 2501 bp from the rDNA NTS containing highly repetitive sequences according to hybridization studies [16,30]. The detailed strategy used for the sequencing of this part of the rDNA in our pRr56 clone is shown in Figure 1. The established nucleotide sequence is presented in Figure 2.

Sequence of downstream rDNA NTS region. The rDNA subcloned in pRr151, pRr22 and pRr24 contains the 3'-terminal part of the 28 S rRNA gene and about 4 kb of the adjacent NTS region. The sequence of the 594 bp rDNA constituting the 3'-terminal part of the 28 S rRNA gene was reported earlier [31]. Here we report the complete sequence (encompassing 4025 bp) of the downstream rDNA NTS shown earlier to contain a SalI tandem repeat region [15] and highly repetitive sequences [15,21,30]. The sequencing strategy for the pRr151 insert

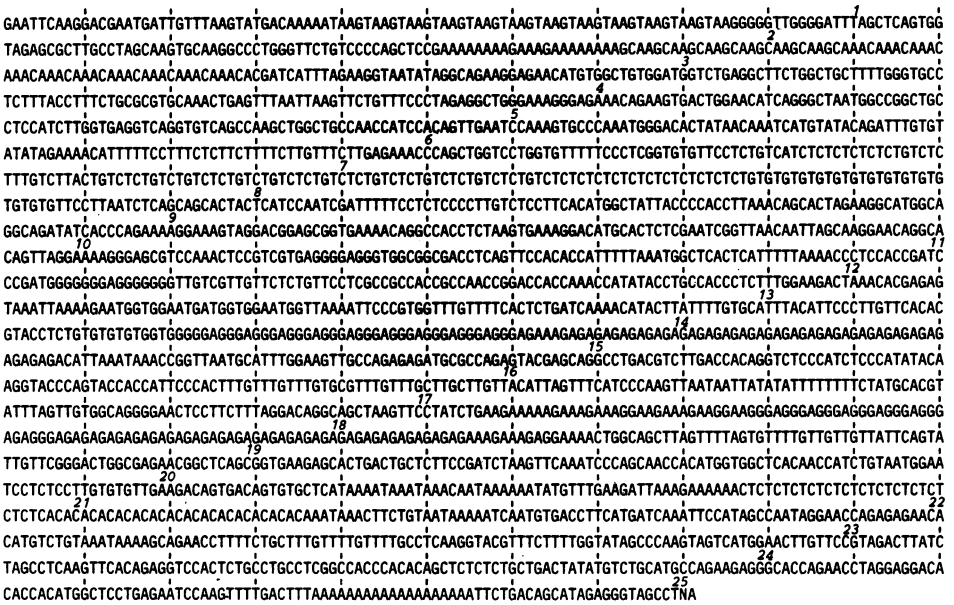

Figure 2. Primary structure of the rat rDNA NTS located upstream from the rRNA transcription unit. The sequence of the EcoRI - SauI subfragment of the pRr56 rDNA is represented (see Fig.1). The numbering is in hundreds starting from the EcoRI site.

is given in Figure 3. The established nucleotide sequence is presented in Figure 4.

Short interspersed sequences in NTS. The comparative computer analysis of our rat rDNA NTS sequences with a DNA sequence bank (EMBL-Heidelberg) allowed us to identify six different members of the B2-superfamily [32-34] (Table 1), while homologies with B1 [32], L1 [35] and U1-U6 snRNA [36] were not found.

Three of the identified short interspersed sequences are located in the upstream (U) and three in the downstream (D) NTS regions. The two proximal B2-like sequences identified in the upstream and downstream NTS possess 85 % homology to the mouse and rat B2 consensus sequence [32,34] and are oriented in opposite directions. Direct or inverted repeats flanking these two B2-like elements cannot be discerned. The two distal B2-like sequences are also located in the upstream and downstream NTS respectively. They are markedly more diverged and are oriented in the same direction (see Table 1).

Two another short interspersed sequences could be identified as corresponding to the rat ID sequence [33]. These repetitive elements are the most

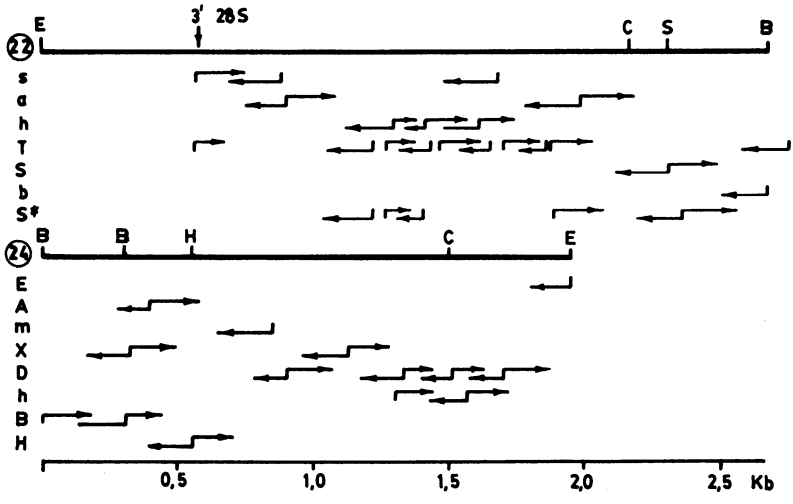

Figure 3. Extended maps and sequencing strategy used in the analysis of the rDNA insert in pRr151 containing the 3'-terminal part of the 28 S rRNA gene [31] and the adjacent downstream NTS region. For convenience the pRr151 constituents pRr22 and pRr24 (plus the BamHI-BamHI subfragment) are represented separately (see Fig.1). The horizontal arrows indicate the direction and the size of sequenced rDNA subfragments obtained with different restriction endonucleases. Only the sites generating the rDNA subfragments used in sequencing are indicated. E-EcoRI; C-Cfr6I; S-SauI; B(b)-BamHI; s-Sau3A; a-AvaII; h-HinfI; T-TaqI; S*-SalI; A-AvaI; m-MvaI; X-XhoI; D-DdeI; H-HindIII

remote from the rRNA transcription unit in both the upstream and downstream NTS segments (see Table 1). The two ID sequences are highly conserved and oriented in opposite directions. They are practically identical to each other and to the consensus ID sequence [33]. The downstream ID sequence is flanked by perfect 15 bp direct repeats (see Fig.4). The upstream ID element is flanked by simple sequences constituted by GTAA or GCAA units. It is possibly significant that the simple sequence AATG is represented twice in each of the direct repeats flanking the downstream ID element.

SalI tandem repeat region. Earlier restriction endonuclease analysis has identified SalI tandem repeats in the downstream NTS region [15]. Determination of the nucleotide sequence in this region confirmed the presence of 6 SalI sites in the rDNA segment encompassing positions D 630 to 1290 (see Fig.4). However, clearly expressed tandem repeats are absent. The detailed computer search for homology allowed the identification of highly diverged short tandem repeats encompassing the conserved SalI sites. Despite the pre-

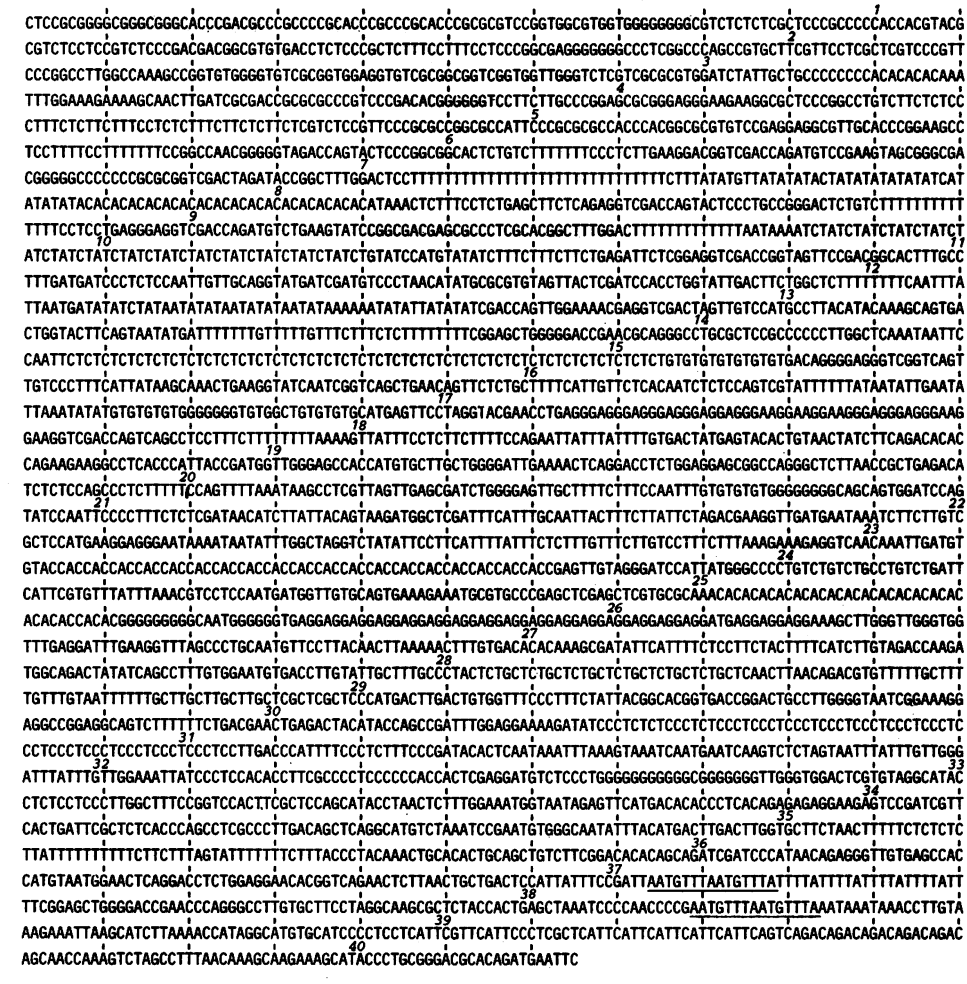

Figure 4. Primary structure of the rat rDNA NTS located downstream from the 3'-terminus of the 28 S rRNA gene [31]. The sequence of the NTS part of the EcoRI - EcoRI rDNA subfragment in pRr151 is represented (see Figs 1 and 3). The numbering is in hundreds starting from the first nucleotide pair adjacent to the 3'-terminus of the 28 S rRNA gene. The 15 bp direct repeat flanking the identified ID sequence is underlined. The two BamHI sites within pRr151 delineating the rDNA inserts in pRr22 and pRr24 (see Fig.3) are at positions 2083-2088 and 2382-2387 respectively.

sence of numerous deletions and insertions of simple sequences [$(AT)_n$, $(AC)_n$, $(ATCT)_n$] it is possible to discern the presence of characteristic T-stretches at the repeat borders and of distinct TC motifs preceding the SalI sites.

Table 1. B2 superfamily elements in the NTS of the rat rDNA repeating unit

N°	Type	Coordinates*	Length (bp)	Percent homology with B2 or ID consensus	Orientation**
1	ID	U 87 - 182	96	98	→
2	B2	U 806 - 957	152	51	←
3	B2	U 1877 - 2066	190	85	→
4	B2	D 1799 - 1992	194	85	←
5	B2	D 3524 - 3703	180	65	←
6	ID	D 3719 - 3817	99	98	←

* The position number is as given in Figures 2 and 4. NTS positions: U - upstream and D - downstream from the rRNA transcription unit.
** ——→ : Orientation of element coincides with direction of transcription

Simple sequences and poly(purine)·poly(pyrimidine) stretches. Both NTS regions are characterized by the presence of numerous simple sequences and rather long poly(purine)·poly(pyrimidine) stretches (only Pu or Py in one strand). The simple sequences found in the NTS segments studied are listed in Table 2.

The analysis of the dinucleotide simple sequences frequency reveals that from the four possible types, practically only two [$(AC)_n/(TG)_n$ and $(AG)_n/(TC)_n$] are present. In contrast, from the two possible self-complementary dinucleotide simple sequences only a relatively short and imperfect $(AT)_n$ segment is found in the SalI repeat region of the downstream NTS (D 747-776), while $(GC)_n$ simple sequences are not present in the whole length of both NTS studied. Among the markedly larger possible number of trinucleotide simple sequences only two types [$(AGG)_n/(TCC)_n$ and $(ACC)_n/(TGG)_n$] could be identified. Only a few of the possible tetra-, penta- and hexa-nucleotide simple sequences are present in the two NTS regions (see Table 2). It is noteworthy that the tetranucleotide simple sequence pairs $(TAAG)_n$:$(ATTC)_n$ and $(AGGG)_n$:$(TCCC)_n$ are in fact identical.

Numerous poly(purine)·poly(pyrimidine) stretches (>20 bp) are observed in both the upstream (8) and downstream (12) NTS. Their formation is more often due to the presence of long mono-,di-, tri- and tetra-nucleotide simple sequences. Some of the poly(purine)·poly(pyrimidine) stretches are rather long. The two poly(purine) stretches in the upstream NTS are of particular interest, encompassing 98 (U 1340-1437) and 124 (U 1706-1829) bp. Most of their length is constituted by the simple sequences $(AG)_n$ and $(AGGG)_n$ comple-

Table 2. Simple sequences in the NTS of the rat rDNA repeating unit

Type*	Simple sequence	Number of units (n) in stretch** U	D	Total number of units in NTS
Mono-	$(A)_n/(T)_n$	21:19	30:14:13:10	107
	$(G)_n/(C)_n$	15	19	34
Di-	$(AC)_n/(TG)_n$	15:5:16	5:17:7:20	85
	$(AT)_n$	--	14	14
	$(GC)_n$	--	--	--
	$(AG)_n/(TC)_n$	8:12:26:24:14	35	119
Tri-@	$(AGG)_n$	--	19	19
	$(ACC)_n$	--	20	20
Tetra-@	$(TAAG)_n$	12	--	12
	$(ATTC)_n$	--	5	5
	$(AGGG)_n$	10:7	8	25
	$(TCCC)_n$	--	14	14
	$(CAAA)_n$	10	--	10
	$(CAAG)_n$	6	--	6
	$(ATCT)_n$	--	15	15
	$(CAGA)_n$	--	5	5
Penta-@	$(CTCTG)_n$	--	6	6
	$(ATTTT)_n$	--	5	5
Hexa-@	$(CTCTGT)_n$	7	--	7

*Only stretches of more than 10 single nucleotides and more than 5 di-, tri- etc. nucleotides are included. Single bp substitutions tolerated. **U - upstream; D - downstream NTS.
@ - Sequence in "non-coding" strand given.

mented by extended AG motifs. The longest poly(purine)·poly(pyrimidine) stretches in the downstream NTS encompass 72 (D 1434-1505), 72 (D 2555-2621) and 71 (D 3037-3107) bp constituted mainly by the simple sequences $(TC)_n$, $(AGG)_n$ and $(TCCC)_n$, respectively.

Generally, the expanded simple sequence and poly(purine)·poly(pyrimidine) stretches constitute about 24 % of the upstream and 22 % of the downstream NTS sequences.

DISCUSSION

The results obtained in the present work permit to elucidate some basic characteristics of NTS organization in the mammalian rDNA repeating unit. Typical for the rDNA of all eukaryotes studied till now is the presence of

relatively short unique sequences flanking directly the transcription start site and the putative transcription termination site near the 3'-end of the 28 S rRNA gene [see 2]. Recent in vitro and in vivo studies with the mouse provide evidence that RNA polymerase I terminates transcription within the rDNA spacer at 565 bp downstream from the 3'-end of the 28 S rRNA gene [37]. Our experiments with the rat have identified also an in vivo rDNA transcript mapping within the T-tract of the downstream spacer at position D563-565 [38]. These results suggest that the rDNA NTS region up to about 0.55 kb downstream from the 3'-end of the 28 S rRNA gene is in fact transcribed by RNA polymerase I. It is noteworthy that while the downstream GC-rich rDNA region is widely diverged in the mouse and the rat, the SalI tandem sites and their immediate vicinity appear to be conserved [37,this work]. The possible role of the proximal SalI site (D 632-637) sequence as an RNA polymerase I transcription termination signal [37] remains to be verified for the rat.

Both upstream and downstream from the rRNA transcription unit the rat NTS is characterized by the presence of tandem repeat regions. The upstream MspI tandem repeats are rather homologous (about 80 % homology among each other) and the different recombinant phages studied contain a varying number of repeats [15,29]. The downstream SalI tandem repeats studied here are markedly more diverged. Restriction endonuclease analysis of an independent rat rDNA clone showed the presence in this NTS region of a single SalI site [21] thus supporting the possibility that extensive structural changes in this NTS region may be tolerated. These features suggest that a short SalI site related sequence may be sufficient to act as a transcription termination signal [see above]. Generally, it seems that the presence of tandem repeat regions in the vicinity of the rRNA transcription unit is a characteristic feature of the rDNA NTS in higher eukaryotes since analogous more or less conserved tandem repeats have been identified in Drosophila [5,6], Xenopus [7], mouse [13,14,37] and human [17] rDNA NTS.

The nucleotide sequences of large NTS regions reported in this work allow us to clarify partly the structural basis for the large increase in NTS size typical for the mammalian rDNA repeating unit [see ref.2]. Basically, the increase in NTS is largely due to the appearence and expansion of a novel type of repetitive sequence: simple sequences combined with short mobile elements. Different types of simple sequences are identified starting from single nucleotide stretches up to penta- and hexa-nucleotide simple repeats (see Table 2). It is remarkable that the different nucleotide combinations are not randomly represented. A markedly prevalent number of simple sequences [$(AG)_n$,

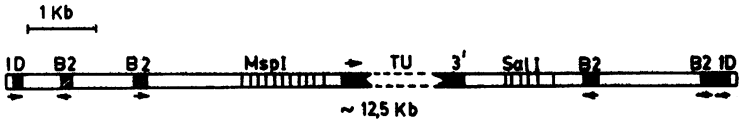

Figure 5. Schematic presentation of the organization of the rat rDNA NTS and the topology of B2-like (B2) and ID (ID) elements. TU - transcription unit. MspI and SalI - tandem repeats in NTS.

$(TC)_n$, $(AGG)_n$, $(AGGG)_n$, $(TCCC)_n$] contribute to the formation of rather long poly(purine)·poly(pyrimidine) stretches in both the upstream and downstream NTS. Further, numerous $(AC)_n/(TG)_n$ simple sequences that may adopt a Z-DNA conformation [39] are present in both the upstream and downstream NTS, a finding that supports directly the conclusions reached in recent hybridization studies with mouse rDNA [40]. In contrast, self-complementary simple sequences are practically absent from the whole NTS length studied. These results suggest that the formation of simple sequences in the rDNA NTS is not random, but reflects the action of some function related genetic pressure.

Six members of the B2 superfamily have also been acquired by the rat NTS, including four B2-like and two ID elements. The sequence analysis reveals that both ID sequences are practically identical to the consensus ID sequence [34] and the downstream sequence is flanked by direct repeats. These data clearly show that the ID repeats have been inserted in relatively recent times into the rat rDNA repeating unit. The four B2-like sequences belong to distinct groups by their degree of homology to the consensus B2 sequence. The proximal B2-like elements are apparently more recent since they display about 85 % homology and they are mainly responsible for the stringent conditions hybridization with an individual recombinant plasmid containing the B2 sequence [30]. The following features of the organization of B2 superfamily elements in the rat rDNA NTS are noteworthy (Figure 5): (a) the B2-like and ID mobile elements appear to be inserted in pairs into the upstream and downstream NTS region; (b) the position of the pairs of mobile elements is roughly symmetric in relation to the MspI and SalI tandem repeat regions; (c) the upstream and downstream B2 superfamily elements are oriented in opposite directions,except for the most diverged upstream B2-like element, and (d) insertion of B2 superfamily mobile elements into the rDNA NTS seems to be selective since other highly repetitive mobile elements (i.e. B1-like sequences) are not found in the analyzed large NTS segments. These observations suggest that the presence of the B2 superfamily elements in the NTS is not fortuituous, but may be re-

lated to some, yet unknown but important, function. Alternatively, the observed insertion pattern of the B2 superfamily elements may reflect the structural organization of the rDNA repeating unit in nucleolar chromatin.

Summarizing it may be stated that the rat rDNA NTS regions flanking the rRNA transcription unit are characterized by the presence of a novel type combination of short interspersed sequences (B2 superfamily) with different types of simple sequences and long poly(purine)·poly(pyrimidine) stretches. These features distinguish the NTS of the mammalian rDNA repeating unit from that of lower vertebrates as exemplified by the NTS of Xenopus [7]. The function of these distant NTS regions remains to be elucidated.

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