A role for upstream binding factor in organizing ribosomal gene chromatin

Jane E. Wright, Christine Mais, José-Luis Prieto and Brian McStay

Biomedical Research Centre, Ninewells Hospital and Medical School, University of Dundee, Dundee DD1 9SY, Scotland, UK.

Abstract

Human ribosomal genes are located in NORs (nucleolar organizer regions) on the short arms of acrocentric chromosomes. During metaphase, previously active NORs appear as prominent chromosomal features termed secondary constrictions, which are achromatic in chromosome banding and positive in silver staining. The architectural RNA polymerase I transcription factor UBF (upstream binding factor) binds extensively across the ribosomal gene repeat throughout the cell cycle. Evidence that UBF underpins NOR structure is provided by an examination of cell lines in which large arrays of a heterologous UBF binding sequences are integrated at ectopic sites on human chromosomes. These arrays efficiently recruit UBF even to sites outside the nucleolus, and during metaphase form novel silver-stainable secondary constrictions, termed pseudo-NORs, that are morphologically similar to NORs.

Introduction

In most eukaryotes, the sole function of Pol I (RNA polymerase I) is the transcription of genes encoding 18S, 5.8S and 28S rRNAs. Like Pol II and Pol III, it requires auxiliary factors that mediate promoter recognition, PIC (pre-initiation complex) formation and transcription initiation. PIC formation on ribosomal gene (rDNA) promoters requires the synergistic action of UBF (upstream binding factor) [1] and the promoter selectivity factor, termed SL1 in humans [2]. SL1 is composed of TBP (TATA-binding protein) and the TBP-associated factors, TAF110, TAF63 and TAF48 [3]. SL1 interacts

To whom correspondence should be addressed (email brian.mcstay@cancer.org.uk).
with promoter DNA in a highly sequence-specific manner, as illustrated by the species-specific nature of rDNA transcription [4]. PICs recruit an initiation competent subfraction of Pol I, defined by the presence of TIF (transcription intermediary factor)-IA/Rrn3 [5,6]. Although clearly involved in promoter function, UBF performs additional roles. After a brief review of the chromosomal organization of human rDNA, we will focus here on the role of UBF in organizing ribosomal gene chromatin.

**NOR (nucleolar organizer regions)**

The human rDNA repeat is 43 kb in length, 13 kb of which is transcribed to yield the 47 S pre-rRNA. The remaining 30 kb is termed the IGS (intergenic spacer) (Figure 1). Approximately 400 copies of the rDNA repeat are distributed among the short arms of the human acrocentric chromosomes (13, 14, 15, 21 and 22) [7], each of which consists of three bands: p11, p12 and p13. Band p12 contains on average 3 Mb of tandem rDNA repeats [8] and is termed the NOR, while p11 and p13 are composed of satellite sequences packaged as heterochromatin. During metaphase, NORs that were transcriptionally active in the previous interphase form prominent chromosomal features termed secondary constrictions, which appear as achromatic gaps when stained with Giemsa or AT-specific fluorochromes such as DAPI (4′,6-diamidino-2-phenylindole) or quinacrine [9]. Electron tomography revealed that chromatin within secondary constrictions is 10-fold less condensed than the rest of the chromosome [10]. Classically, secondary constrictions are positive in silver staining, and are

![Structure of human ribosomal gene repeat and the chromosomal location of NORs](image)

*Figure 1 Structure of human ribosomal gene repeat and the chromosomal location of NORs.* See text for further details.
often termed AgNORs [11]. Association of the transcription machinery with rDNA during mitosis correlates with the presence of a secondary constriction [12,13]. It is generally considered, but not proven, that some component of the transcription machinery, or indeed the act of transcription itself, is responsible for the undercondensation of NORs during metaphase.

**Binding of UBF to rDNA**

UBF belongs to the sequence-non-specific class of HMG (high-mobility-group) box proteins [14] (Figure 2). Unlike other HMG box proteins, UBF dimerizes in solution through sequences in its N-terminus [15]. UBF contains multiple HMG boxes and, like other HMG proteins, interacts with the minor groove of DNA and binds to structured nucleic acids such as cruciforms, or four-way junctions [16]. Like other HMG proteins, UBF induces bending upon binding to non-structured sequences. Moreover, the tandem HMG boxes enable a single dimer of UBF to organize promoter DNA into a 360° loop [16,17]. This so-called architectural ability of UBF, its abundance (approx. 5×10^5 molecules per cell) and its low sequence specificity in DNA binding were a strong indicator of other roles for UBF. Further support for this contention comes from the observation that mammalian cells contain approximately equal amounts of two UBF forms as a result of splice site variation. UBF2 differs from UBF1 in missing 37 residues

![Figure 2 Structure of UBF and its distribution across the human rDNA repeat.](image)

UBF1 and UBF2 are shown in cartoon form. UBF1 comprises 764 amino acids with four HMG box DNA-binding motifs, a dimerization domain at its N-terminus and a highly acidic C-terminus. In the splice variant UBF2, 37 amino acids are deleted from HMG box 2. The relative distribution of UBF across the human rDNA repeat is shown in the lower panel. This graph was derived from previously published ChIP data [26].
from the second HMG box [18]. This deletion renders UBF2 non-functional at the promoter [19]. These characteristics suggested that UBF has a more general structural role on active NORs.

UBF can bind in vitro to repeated elements that function as rDNA-specific transcriptional enhancers [20,21]. Experiments with in vitro systems demonstrate that UBF is required for enhancer function [22]. Interestingly, UBF from heterologous species and UBF2 can support enhancer but not promoter, function. Further in vitro experiments have shown that UBF can bind across the entire Xenopus IGS [23] and to sequences downstream of the transcriptional start site [24]. However, it should be pointed out that UBF also binds to poly(dI-dC) and vector DNA. Consequently, in vitro binding studies do not provide reliable evidence for extensive binding of UBF to the rDNA repeat in vivo. In order to address the distribution of UBF in vivo, we developed a ChIP (chromatin immunoprecipitation) assay that utilizes an enriched nucleolar chromatin fraction [25]. We demonstrated for the first time that UBF binds in vivo to multiple sites distributed across the entire human rDNA repeat (transcribed sequences and IGSs) [25,26] (Figure 2). Despite this diversity of binding sites, UBF binds selectively to rDNA, as it is localized exclusively to nucleoli during interphase and at NORs during mitosis [27]. We have proposed a model in which rDNA repeats contain specialized high-affinity binding sequences that bind UBF in a co-operative manner and induce spreading of UBF on to adjacent lower-affinity binding sites across the rDNA repeat [25].

Effects of UBF binding

The next question was to determine the consequences of this extensive UBF binding. Since UBF remains bound to NORs during mitosis, we were particularly interested in determining if extensive binding of UBF is responsible for both the undercondensation and silver staining of NORs in metaphase. The strategy we employed to address this question was to generate cell lines in which large arrays of a heterologous UBF-binding sequence were integrated into ectopic sites on human chromosomes [26]. The sequences employed, XEn, were blocks of 60/81 bp repeats present in the IGS of Xenopus rDNA (Figure 3A). These are well characterized UBF-binding sites [28] and function in a UBF-dependent manner as transcriptional enhancers [22]. Despite a lack of sequence identity with human rDNA, it was already known that human UBF binds to these elements in vitro [29]. We have characterized eight independent cell lines in which arrays of XEn ranging from 100–2000 kb have been integrated at various chromosomal sites. In Figures 3(B) and 3(C) we show data from clone 3D, in which 1400 kb was integrated into the long (q) arm of human chromosome 10. Combined immuno-FISH (fluorescence in situ hybridization) was utilized to demonstrate that these arrays bind UBF during interphase, independent of nuclear location and chromosomal integration site (Figure 3B). Analysis of chromosomes demonstrated that UBF remains bound to XEn arrays during metaphase and is associated with the presence of a novel secondary constriction, particularly evident in reverse DAPI banding (Figure 3C). The XEn array in
this clone represents approx. 1% of the DNA present in this chromosomal arm. Notably, the novel secondary constriction associated with these sequences represents a significantly larger fraction of the contour length of the metaphase chromosome. Presumably this is a consequence of its undercondensed state.

The second primary characteristic of metaphase NORs is that they are positive in silver staining. It was generally assumed that this was due to the presence of an abundant argyrophilic protein. The acidic C-terminus of UBF is a prime candidate for silver binding. The novel secondary constriction on chromosome 10q in clone 3D is clearly positive in silver staining (Figure 4A). Note also that the XEn array is clearly visualized by silver staining during interphase (Figure 4B). We conclude from these experiments that recruitment of UBF to XEn arrays results in formation of a chromatin structure with all the primary characteristics of NORs. We refer to these structures as pseudo-NORs, since they do not organize formation of a nucleolus. The precise role of UBF in forming secondary constrictions is not yet clear to us, but we can now propose a number of conclusions. First, as pseudo-NORs are inactive, due to the absence of promoter sequences, transcription in the previous interphase

Figure 3 UBF binds to ectopic binding site arrays and results in formation of a novel secondary constriction termed a pseudo-NOR. (A) The plasmid pXEn8mer was constructed which contains eight blocks of the enhancer element or eighty 60/81 bp repeats (red boxes). The pXEn8mer insert was transfected into a human fibrosarcoma cell line, HT1080, together with a blastcidin resistance marker. (B) Combined immuno-FISH was performed on interphase cells of clone 3D. UBF was visualized using FITC-conjugated affinity-purified antibodies. XEn arrays were visualized with a spectrum red-labelled probe. DIC, differential interference contrast (C) Combined immuno-FISH was performed on metaphase spreads prepared from clone 3D. UBF and XEn were visualized as above. Left panels show standard DAPI staining; right panels show enhanced reversed DAPI staining.
is not a pre-requisite. Secondly, the ability to form secondary constrictions is intrinsic to rDNA. The de novo formation of pseudo-NORs at ectopic sites demonstrates that sequences distal and proximal to NORs are not required. Finally, binding sites for other components of the Pol I transcription machinery are not required, as UBF-binding sites are sufficient to specify this structure.

Micrococcal nuclease digests of nuclei prepared from clone 3D clearly demonstrate a classical nucleosomal ladder associated with XEn sequences (Figure 4C). The nature of these nucleosomes is under investigation. The key question, however, is how does extensive UBF binding result in undercondensation and formation of a secondary constriction during metaphase? One possibility is that UBF binding to nucleosomal DNA inhibits binding of linker histones required for chromatin condensation. In this regard, it is worth pointing out that UBF can bind in vitro to nucleosomes with high affinity and displace linker histone H1 [30].

**Figure 4** Pseudo-NORs are positive in silver staining and packaged as nucleosomes. (A) Metaphase spreads prepared from clone 3D were both DAPI-stained and-silver stained. Pseudo-NORs are indicated by arrowheads, and true NORs by arrows. (B) Staining of a clone 3D interphase nucleus by DAPI and silver. The pseudo-NOR is indicated by an arrowhead. (C) Nuclei from clone 3D were digested with an increasing concentration of micrococcal nuclease. DNA was recovered, Southern-blotted and probed with human repetitive DNA sequences (COT1) DNA to visualize bulk DNA (left panel) or XEn DNA (right panel).
Conclusions and perspectives

Although highly conserved among vertebrate organisms, UBF is not present in invertebrates, fungi or plants. A role for HMG box proteins in rDNA chromatin organization does, however, appear to have been conserved throughout evolution. Hmo1p, a nucleolar protein with a single HMG box, has some sequence similarity to UBF and strongly enhances rDNA transcription in yeast [31]. This may explain why secondary constrictions can be observed associated with the NORs of non-vertebrate organisms.

We have observed that, during interphase, pseudo-NORs sequester a significant fraction of every component of the Pol I transcription machinery, even to sites outside nucleoli and independent of both promoter sequences and transcription [26]. These results have forced us to re-evaluate how the Pol I machinery is recruited to nucleoli and rDNA. Moreover, it appears that maintenance of rDNA chromatin in an undercondensed state throughout mitosis facilitates the rapid onset of transcription in the subsequent G₁ stage of the cell cycle.

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References