Putative involvement of the histone acetyltransferase Tip60 in ribosomal gene transcription

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ABSTRACT

Tip60 is a histone acetyltransferase (HAT) implicated in a wide range of cellular functions, including mRNA synthesis and DNA repair. In the present report we propose a model based on which Tip60 is actively involved in ribosomal gene transcription through acetylation of UBF, a ribosomal specific transcription factor, as well as through its direct recruitment to the human ribosomal gene promoter, as shown by chromatin immunoprecipitation experiments. Electron microscopy studies revealed that Tip60 resides in sites of active rDNA transcription within the nucleolus, while it co-localizes with UBF as shown by confocal microscopy. In addition, *in vivo* transcription assays demonstrated that the nucleolar fraction of Tip60 localizes to sites of newly synthesized rRNA. Finally, functional assays established that Tip60 complexes with, and targets UBF for acetylation. The present study underlines the importance of acetylation in rDNA transcription and directly implicates Tip60 in the process of ribosomal gene transcription.

INTRODUCTION

Although not all ribosomal DNA (rDNA) repeats are utilized at any given moment, pre-rRNA synthesis is a highly active process and rRNA can constitute up to 80% of total RNA in a cell (1). While the mechanisms maintaining the ratio of active versus silent rDNA repeats are not yet clarified, different histone modifications have been implicated in the establishment of a euchromatic or heterochromatic gene structure (2). Chromatin remodelling is known to be facilitated by the recruitment of co-activators bearing histone acetyltransferase (HAT) activity (3–5) by which they alter the structure of the nucleosome and allow RNA polymerase II (RP II) mediated gene transcription. However, the detailed mechanism leading to the transition from the inert nucleosomal state of ribosomal genes to enhancesome formation (6), has not yet been determined. The regulation of RNA polymerase I (RP I)-mediated transcription (reviewed in 7) by protein acetylation,

through the competitive recruitment of HAT CBP and Rb, has been recently demonstrated (8), while the overall effect of acetylation upon ribosomal transcription has been investigated (9). In both these reports, acetylation is shown to affect rDNA transcription both directly, by histone acetylation of lysine residues and chromatin remodelling, and indirectly, through the recruitment and targeting of the RP I-specific, architectural transcription factor, UBF (upstream binding factor) (10–13).

Tip60, a MYST family HAT first identified as an HIV-Tat interacting protein (14), is an androgen receptor (AR) co-activator (15,16) and exerts its effect via directly acetylating the AR, while it counters the deacetylating repressing effect of HDAC1 (17).

In this study, we show that Tip60 is partially localized in the nucleolus of prostate cancer (CaP) cell lines and is actively involved in rDNA transcription both by complexing with, and acetylating UBF, and by being recruited, along with UBF, onto the human rDNA gene promoter upon hormone stimulation. Using *in vivo* transcription assays and electron microscopy, we demonstrate that Tip60 resides within sites of active rRNA synthesis. Our results implicate Tip60, a HAT enzyme and steroid receptor co-activator, in the transcription of ribosomal genes, and present a potential mechanism underlying its involvement in rRNA synthesis.

MATERIALS AND METHODS

Plasmids

The Tip60 constructs used in the present study, including the HAT-deficient mutant Tip60$^{Q377E/G380E}$, have been described (16–18).

Cell culture and drug treatment

The human CaP cell lines LNCaP and PC3M (AR positive and negative, respectively) and COS-7 monkey kidney cell line were cultured in RPMI 1640 media (Sigma) supplemented with 10% fetal calf serum, 1% glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml) (referred to as Full Medium; FM). Steroid depleted medium (SDM) was made as previously described (18) and is hormone and growth factor depleted. Trichostatin A (TSA), actinomycin D, DRB, α-amanitin and RNase were purchased from Sigma.

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Generation of FLAG-Tip60 inducible cell line

FLAG-Tip60 full-length cDNA was produced as described above, then cloned into the *Eco*RI site of pcDNA4.1 (Invitrogen). This was then co-transfected into PC3M cells with pcDNA6 encoding Tetracycline repressor. Transfected cells were selected using Blasticidin and Zeocin (Invitrogen), clones were expanded and tested for inducibility by exposure to 1 µg/ml doxycycline and western blotting using anti-FLAG M2 antibody. The clone used in this study exhibited no leaky expression and showed strong induction of FLAG-TIP60 protein with doxycycline exposure.

Antibodies

Anti-Tip60, anti-Ac-Lys, non-specific IgG and anti-AcH4 antibodies were obtained from Upstate. Anti-CBP, anti-SC35 and anti-UBF antibodies were from Santa Cruz and anti-FLAG antibody was from Sigma. Anti-fibrillarin and anti-nucleolin antibodies were from Cytoskeleton and Research Diagnostics, respectively. The antibody directed against rRNA was developed by Ethan Lerner (19) and was a gift from J. Steitz (20). The anti-B23 antibody was a gift from D. Hernandez-Verdun. Anti-RP53 antibody was kindly provided by I. Grummt. The anti-BrUTP antibody was obtained from Roche. Secondary anti-rabbit and anti-mouse antibodies Alexa 488 and 633 nm were from Molecular Probes. The FITC conjugated secondary was obtained from Dako.

Western blotting and co-immunoprecipitation experiments

Sample preparation, electrophoretic separation, western blotting and co-immunoprecipitations were performed as described (15).

Chromatin immunoprecipitation assays (ChIP)

ChIP assays were performed as previously described (17). A DNA fragment containing the promoter region of human rRNA gene including the upstream control element (UCE) was amplified by PCR using primers: F: (5′-CCCCTGC-GTGTGGCACGGGC) and R: (5′-AGGAGCGCGGCC-GGCTAGCC) as described (21). The PCR reaction was performed using 25–35 cycles, each cycle comprising of 94°C, 30 s; 60°C, 20 s; and 72°C, 30 s. Radiolabelled products were resolved on 4% polyacrylamide gel, dried and exposed to autoradiography.

Immunoelectron microscopy

Electron microscopy immunogold staining was based on the method described (22). A semi-quantitative analysis was performed to assess Tip60's sub-nucleolar localization (Table S1). For a detailed description of the methods used, see Supplementary Material.

In vivo run-on transcription in permeabilized cells

Run-on transcription was performed as previously described (23), and modified (24) to favour detection of RNA polymerase I-mediated transcription. For control experiments, no BrUTP incorporation was detected in each of the following cases: (i) no primary antibody; (ii) UTP replaced BrUTP in transcription buffer; (iii) RNase digestion (50 µg/ml) for 10 min following *in vivo* transcription; (iv) actinomycin D (5 µg/ml) added to transcription cocktail to inhibit transcription. When α-amanitin was used to block RNA polymerase II- and III-mediated transcription (100 µg/ml), an exclusively nucleolar signal was detected, verifying that nucleolar incorporation of BrUTP was due to RNA polymerase I transcription only.

Protein purification

Recombinant (His)$_6$-tagged Tip60, UBF (kind gift from Joost Zomerdijk) and PCAF were overexpressed in insect cells and purified to >95% using Ni^{2+}-affinity chromatography according to the manufacturer's guidelines (Qiagen). Enzymatically active Tip60 and PCAF were confirmed using histone substrates.

In vitro acetylation assays

Purified UBF and PCAF or Tip60 from Sf9 cells were incubated in 30 µl of HAT buffer [50 mM Tris–HCl (pH 8), 0.5 mM EDTA, 10% glycerol and 20 µM acetyl-CoA] for 30 min at 30°C. Reactions were terminated upon addition of SDS sample buffer. Acetylation was detected by western blotting with anti-acetylated lysine antibody.

To assess Tip60's acetyltransferase activity and substrate specificity, crude histones or bovine serum albumin (BSA) were incubated with recombinant Tip60 in the presence of 3H-acetyl CoA for 30 min. Products were resolved by SDS–PAGE, dried and subjected to fluorography.

Immunofluorescence, digital image processing and data quantification

Immunofluorescence was performed as described (18). For CBP, paraformaldehyde fixation was undertaken and the antibody incubations were performed at 37°C for 30 min–1 h (9). A series of 1 µm vertical optical sections through the entire thickness of the tissue was used to produce a *z* series. Blind deconvolution (reviewed in 25), an iterative-constrained algorithm capable of improving the resolving power was performed using AutoDeblur software, to improve *z*-axis resolution (final resolution: 0.5 µm). Background equalization was performed using the AutoVisualize application. By running our confocal image stack through the AutoDeblur Blind Deconvolution software, we avoided signal attenuation due to photobleaching. The latter was achieved by using a small number of sections and averages per section, while there was no significant loss of *z*-axis resolution.

To study the intra-nucleolar distribution profile of UBF and Tip60, the 'line quantification' tool of the LCS 2.5 v1104 software was used (18). Dual line quantification allowed assessment of the degree of co-localization between the two signals under different conditions and the correlation coefficient *r* was calculated for each case.

Labelling controls were carried out by omitting the primary antibody. Secondaries conjugated to different fluorescent tags were used on several occasions and exhibited the same signal distribution (Figs 2 and 6).

Flow cytometric analysis

The S-phase fraction was used as a means of assessing cell proliferation as described (26,27). Cells (5 × 10^5) were trypsinized, resuspended in PBS supplemented with 10% fetal calf serum, pelleted and then washed in PBS. Cells were then

incubated for 10 min at 4°C with 100 µg/ml freshly prepared RNase A and stained with 100 µg/ml propidium iodide in TBS/Triton-X 2%. Cell cycle profiles and distributions were determined by flow cytometric analysis of 10 000 events using the Lysis II program on a FACScan flow cytometer (Becton-Dickinson). For the calculation of cell population per cycle phase and the generation of density contours, Cylchred and WinMDI 2.8 software were used, respectively. All experiments were independently performed at least three times.

Statistical analysis

Pearson's Product Moment Correlation Coefficient (r) was calculated to assess the degree of covariance between UBF and Tip60 signal under the different growth conditions. Student's t-test was used to assess the difference between the r values in each case. At least three independent experiments were performed in each case and a P value of less than 0.05 was considered statistically significant. Student's t-test was also applied for the assessment of the FACS data.

RESULTS

Tip60 is partly localized to the nucleolus and responds to hormones and growth factors

Recently, we reported Tip60 to be present in both cellular compartments in prostate tissue and cancer cell lines, while its distribution depends on the presence of hormones and growth factors (18). As shown in Figure 1A, Tip60 is diffusely distributed in the nucleoplasm with the formation of several aggregates when steroids and growth factors are present (FM). Under the above conditions, LNCaP cells are cycling with ~20% of the cell population in the S phase of the cell cycle (see FM contour and corresponding 'dotted' bar chart in Fig. 1D). However, Tip60 accumulates in areas characterized by low DAPI (4,6-diamidino-2-phenylindole DNA dye) signal (blue), in the absence of hormones and growth factors (SDM) in LNCaP cells (Fig. 1B). Other CaP cell lines of different degrees of malignancy (e.g. DU145, PC3, PC3M) displayed a similar nucleolar pattern for Tip60 (data not shown). In SDM conditions, LNCaP cells arrest predominantly at the G1/0 phase of the cell cycle, while the percentage of cells actively synthesizing DNA is decreased to ~2% (SDM contour and 'squared' bar chart in Fig. 1D). Transient and stable transfection with Tip60-RFP (red fluorescent protein) gave similar results to those reported above for endogenous Tip60, in living cells (18).

We also investigated the localization of CBP in CaP cell lines. CBP is a HAT directly implicated in ribosomal gene transcription (8) and endogenous CBP is localized to the nucleolus (9) in mouse embryonic fibroblasts. In AR positive LNCaP cells (Fig. 1C) and androgen independent PC3M cells (data not shown), CBP is homogeneously expressed in the nucleoplasm with an enrichment in bright foci, likely corresponding to PML bodies (reviewed in 28), while it is excluded from the nucleolus. The absence of CBP from the nucleolus has been previously reported (29,30). Our results are suggestive of a cell type specific HAT distribution, with Tip60 potentially providing the major nucleolar HAT activity in the CaP cell lines examined, where CBP is excluded from the nucleolar compartment.

### Tip60's intra-nucleolar localization in interphase cells

To further investigate the localization of the nuclear population of Tip60, an anti-SC35 antibody was employed. SC35 is a conserved pre-mRNA splicing and 'speckle' specific factor, actively involved in the processing of pre-mRNA transcripts. Since Tip60 has been previously implicated in mRNA transcription as a nuclear receptor co-activator (15,16), we were interested in whether this HAT enzyme localizes to those speckles (31,32) where splicing and transcription factors accumulate (reviewed in 33). Considering the mRNA transcription and processing apparatus distribution could depend upon transcriptional activity (34), both cycling and G0 arrested cells were used (Fig. 1D). In both cases, there was no apparent co-localization between Tip60 and SC35 (Fig. 2A and B, respectively).

Based on the DAPI overlay images suggesting Tip60 could be localizing in the nucleolus and the lack of co-localization with SC35, we compared Tip60's distribution with that of nucleolar proteins involved in rRNA processing. Fibrillarin, nucleolin and B23 participating in early, both early and late, and mainly late stages of rRNA processing, respectively, were studied. As demonstrated in Figure 2, Tip60 showed a high degree of co-localization with both fibrillarin (Fig. 2C and D) and nucleolin (Fig. 2E and F), as well as with B23 (data not shown). Nucleolin, which resides in the peripheral granular component of the nucleolus (GC), showed a more spread out pattern compared to fibrillarin (mainly found at the dense fibrillar component; DFC). It also exhibited a higher degree of co-localization with Tip60 when cells were grown in SDM (Fig. 2F), coincident with Tip60 showing the highest nucleolar accumulation. In contrast, we observed a larger overlap between the nucleolar population of Tip60 and fibrillarin, in cycling cells (Fig. 2C).

We further used an antibody directed against ribosomal RNA to investigate whether the nucleolar fraction of Tip60 co-localizes with the ribosomal transcripts. The antibody, isolated from mice with a lupus erythematosus-like syndrome, was shown to specifically recognize an antigenic determinant on the large RNA moiety of the ribosome rather than on a ribosomal protein. As expected, it stained the nucleolus (sites of ribosomal synthesis) and the cytoplasm (19) with the nucleolar signal representing the ribosomal RNA (green) showing a high degree of co-localization with Tip60 (red) (Fig. 2G and H).

Tip60 co-localizes with UBF

Previous studies have demonstrated the important role that histone and UBF acetylation has to play in facilitating ribosomal transcription (8,9). We were interested in the relative localization of Tip60 and UBF, a ribosomal transcription factor residing within the fibrillar centres (FC) of the nucleoli. As illustrated in Figure 3A, there was a high degree of overlap between Tip60 and UBF. Nucleolar co-localization of a HAT enzyme and UBF strongly suggests a potential involvement of Tip60 in ribosomal transcription. Interestingly, Tip60 seems to co-localize with UBF throughout mitosis (Fig. 3B, early metaphase), while there is very little overlap with the early pre-rRNA processing protein fibrillarin at the chromosomal periphery and the pre-nucleolar bodies (PNBs) (data not shown). Since UBF remains associ-
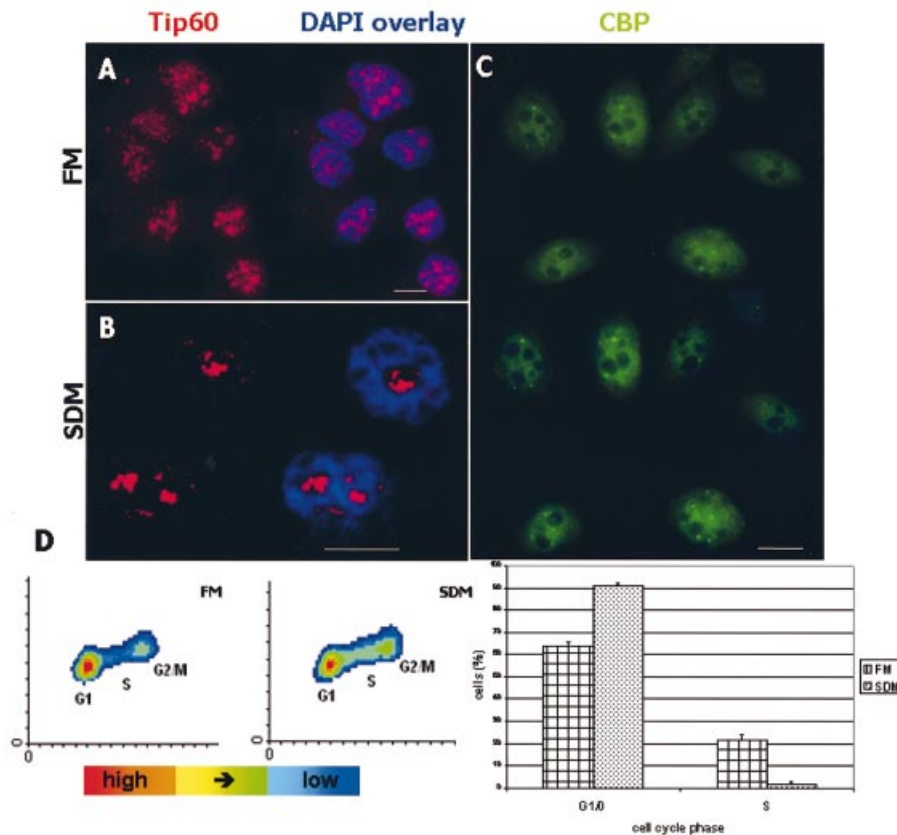
Figure 1. Subcellular distribution of Tip60 and CBP in LNCaP cells. (**A**) In full medium (FM), Tip60 (red) shows a diffuse nucleoplasmic distribution while it accumulates in areas of low DAPI (blue) uptake. (**B**) Tip60's nucleolar localization becomes more apparent when cells are grown in the absence of steroids and growth factors (SDM), while the nucleoplasmic signal completely disappears. (**C**) CBP (green), is absent from the nucleoli of LNCaP cells. Bar = 30 μm. (**D**) Flow cytometric analysis. Cell density contours illustrating the distribution of LNCaP cells in FM or SDM conditions. Coloured intensity scale corresponding to percentage of cells in the different cell cycle phases (i.e. G1/0, S and G2/M). Bar chart showing the results of the data quantitative analysis. Square pattern: FM; dotted pattern: SDM. The average of three independent experiments is depicted along with S.E. bars. The S phase population is significantly reduced ($P < 0.05$) when culture medium containing hormones and growth factors (FM) is replaced by SDM, that causes cell cycle arrest and accumulation at the G1/0 phase.

ated with rDNA genes within the nucleolar organizer regions (NORs) along with the rest of the RP I transcription machinery (RNA Pol I, TTF-1, SL-1) throughout mitosis (35,36), our data further implicate the nucleolar fraction of Tip60 in ribosomal gene transcription and rRNA synthesis.

Cell starvation has long been correlated with changes in ribosomal transcription (37), while growth factor stimulation has also been shown to enhance the transcriptional response (38). Therefore, we investigated the effect of hormone and growth factor deprivation (SDM) on the degree of UBF and Tip60 co-localization. As shown in Figure 3C, there is co-localization between the two proteins both in cycling (FM) and SDM treated cells. However, the degree of this co-localization (yellow signal in Fig. 3A; overlapping red and green peaks in line-scans in Fig. 3C), as measured by coincident Tip60 (red) and UBF (green) signals of the dual line-scans, depends upon the presence of hormones and growth factors. To further characterize and quantify the level of the above coincidence, Pearson's correlation co-efficient r was calculated. Specifically, dual colour line quantification was performed (X: point co-ordinates, Y: average pixel intensity) on at least five cells, in three independent experiments. Each measurement involved line quantification of at

least 300 points. The correlation Pearson's co-efficient was calculated in each case and the mean $\pm$ S.E. was calculated for each experiment. Student's t-test was performed and the difference between r_{SDM} (0.32 ± 0.07) and r_{FM} (0.87 ± 0.02) was found to be statistically significant ($P = 0.02$). In conclusion, the degree of Tip60's co-localization with UBF decreases when cells are arrested in G0, compared to fully cycling cells. Additionally, there is an apparent translocation of Tip60 towards the periphery of the UBF signal (nucleolar periphery) upon hormone and growth factor deprivation, leading to the observed decrease in the degree of overlap.

Tip60 resides at the cortex of the fibrillar centres

To examine whether endogenous Tip60 localizes to sites of active rDNA transcription, electron microscopy and immunogold labelling were used to analyse its intra-nucleolar distribution in ultrathin cryosections (Fig. 4). As illustrated in Figure 4A, in interphase nucleoli, the DFC appears as a strongly electron-dense structure surrounding a rounded finely fibrillar area corresponding to the FC, while most of the nucleolus is formed by the GC which is of intermediate electron density between the FC and the DFC. This confirmed that the fine ultrastructure seen in epoxy resins (data not
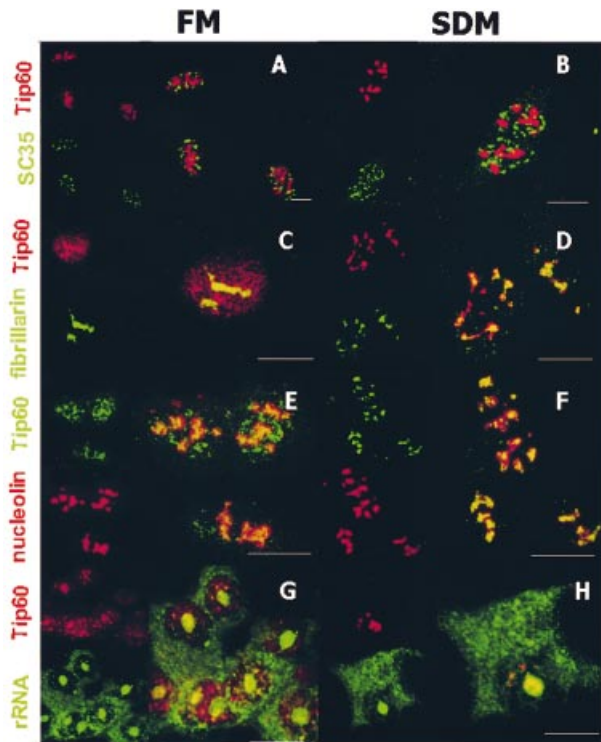
Figure 2. Combined localization of Tip60 and subnuclear compartment markers. (**A** and **B**) Tip60 (red) does not co-localize with SC-35 (green), a pre-mRNA splicing factor, in either FM or SDM. (**C** and **D**) Tip60 (red) shows a high degree of overlap with the dense fibrillar component protein, fibrillarin (green), in both FM and SDM conditions. (**E** and **F**) Similarly, nucleolin (red), a granular component marker, co-localized with Tip60 (green) in FM and SDM, with a stronger yellow signal (overlay) in SDM. (**G** and **H**) Nucleolar rRNA detected using an anti-RNA antibody (green), co-localized with Tip60 (red) under both FM and SDM conditions. FM: full medium; SDM: steroid depleted medium. Bar = 30 μm.

shown), was retained following paraformaldehyde fixation and immuno-labelling (Fig. 4A). We proceeded, therefore, to qualitatively describe Tip60's distribution amongst these different nucleolar compartments after immuno-labelling with 10 nm gold particles.

As shown in Figure 4B, particles were confined to small regions in the cortex of FC close to the surrounding DFC in all cases studied. The GC of the nucleolus remained generally unlabelled, whereas there was a degree of staining detected in the nucleoplasmic area near decondensed chromatin formations (data not shown). We were not able to observe a difference in Tip60's intra-nucleolar distribution pattern between the cells grown in FM and SDM. It is now widely accepted that transcription of rDNA takes place in a transient area between the FC and the DFC (39–41). Therefore, our qualitative electron microscopy results, summarized in Table S1, further support a potential role for Tip60 in rRNA synthesis by localizing this HAT enzyme at the sites of rDNA transcription.

### Tip60 co-localizes with RP I transcription sites in the nucleolus

In situ run-on assays represent an extremely sensitive technique with which to reveal ribosomal transcription (24,42). To obtain further evidence for the functional involvement of
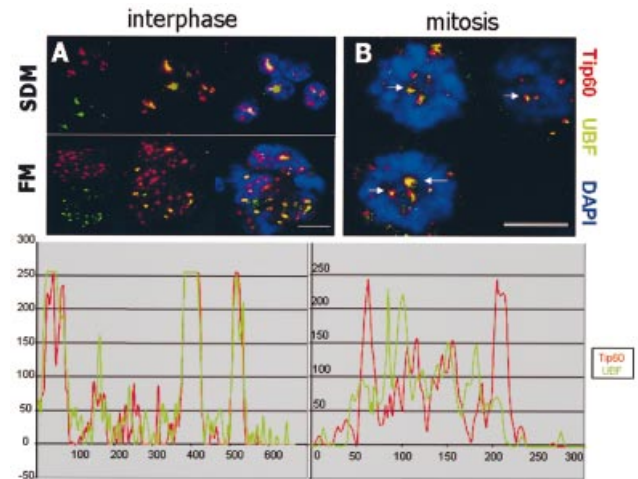
Figure 3. Tip60 and UBF co-localize in the nucleolus. (**A**) Tip60 (red) and UBF (green) co-localize in interphase cells (yellow overlap), in both FM and SDM conditions. (**B**) Tip60 (red) resides within the NORs (arrows) along with UBF (green) in mitotic cells. (**C**) Dual line quantification of the red (Tip60) and green (UBF) signal, in order to measure the degree of overlap under FM and SDM conditions, using the Pearson's correlation coefficient (*r*). As illustrated in (C), cycling cells show a higher degree of co-localization between the two proteins in comparison to steroid factor and growth hormone depleted cells (SDM). FM: full medium; SDM: steroid depleted medium. Single bar = 10 μm, double bar = 1 μm.

Tip60 in rDNA transcription, the relationship of Tip60 to the cellular transcription program in the CaP cell line LNCaP was examined. This experiment was performed in full medium in order to maximize rRNA synthesis and, consequently, Tip60 is detected in both nucleolar and nucleoplasmic compartments. Upon bromo-uridine triphosphate (BrUTP) incorporation and subsequent fluorochrome-conjugated antibody detection (green), sites of both mRNA and rRNA synthesis were revealed throughout the nucleoplasm and the nucleolus, respectively. Since the labelled RNA is predominantly localized at sites of active transcription, the overlap between Tip60 and newly synthesized rRNA in the nucleolus (Fig. 5A, arrows) strongly suggests a potential functional involvement of Tip60 in the process of ribosomal gene transcription.

Both nucleolar and extranucleolar incorporation sites were detected. Under the experimental conditions used, RP III activity is not detected (23), therefore, all transcription visualized in the extranucleolar compartment was mediated by RP II. When α-amanitin was included in the transcription cocktail at a concentration completely inhibiting RP II (100 μg/ml), but not RP I-mediated transcription, the entire nucleoplasmic signal was abolished, leaving only nucleolar incorporation sites (green), again showing a high degree of overlap with Tip60 (red, Fig 5B i). RNase A digestion prior to BrUTP immunodetection abolished all BrUTP labelling (no green, Fig 5B ii) demonstrating that BrUTP is specifically incorporated into RNA. Similarly, high actinomycin D concentrations (5 μg/ml) that inhibit all transcriptional activity, led to complete abolishment of all BrUTP signal (Fig. 5B iii), further verifying that incorporation is due to RNA polymerase activity. The specificity of the anti-BrUTP antibody is shown by the absence of labelling when BrUTP was replaced by UTP in the run-on buffer (Fig. 5B iv).
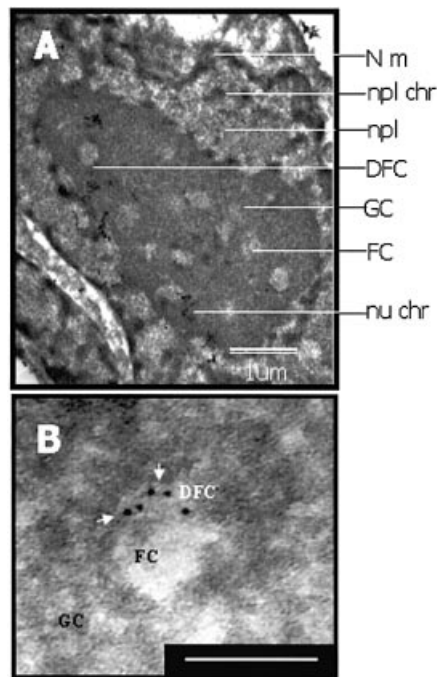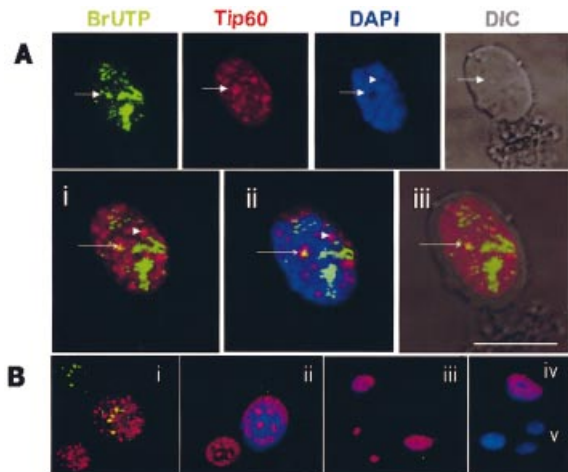
Figure 4. Study of the intra-nucleolar distribution of Tip60 by immuno-gold labelling electron microscopy. (**A**) Preservation of the cellular architecture after paraformaldehyde fixation; the different cellular compartments are shown: N m: nuclear membrane; npl chr: nucleoplasmic chromatin; npl: nucleoplasm; DFC: dense fibrillar component; GC: granular component; FC: fibrillar centre; nu chr: nucleolar chromatin. (**B**) Tip60 localizes in the border area between the FC and the DFC (white arrows). Bar = 200 nm. Also see Supplementary Material, Figure S1.

Figure 5. Tip60 resides within sites of active ribosomal gene transcription. (**A**) *In vivo* run-on transcription assay demonstrating Tip60's co-localization with actively transcribed rDNA. Upper panel: BrUTP incorporation (green) at sites of active rRNA and mRNA transcription throughout the nucleolus and the nucleoplasm; Tip60 (red) diffusely distributed within the nucleoplasm with nucleolar aggregates (arrow); DAPI (blue) DNA dye outlining the nucleolus (arrow and arrowhead); DIC/Normasky image where the basic cellular architecture is illustrated (arrow and arrowhead: nucleolus). Lower panel: (i) red–green overlap; arrows indicate co-localization. (ii) Same as (i) with DAPI overlay showing the nucleolar localization of the yellow co-localization signal (arrows). (iii) Same as (i) with DIC overlay; the arrow illustrates the nucleolus. (**B**) Controls demonstrating the specificity of the assay. (i) RP II inhibition by α-amanitin (100 μg/ml) results in abolishment of the nucleoplasmic signal, leaving only four nucleolar incorporation sites (green) completely overlapping with nucleolar Tip60 (yellow). (ii) RNase A digestion prior to BrUTP immunodetection abolished all BrUTP labelling (no green signal) demonstrating that BrUTP is specifically incorporated into RNA. (iii) Actinomycin D (5 μg/ml) inhibiting all transcriptional activity, led to complete abolishment of BrUTP further verifying incorporation is due to RNA polymerase activity. (iv) When BrUTP was replaced by UTP in the run-on cocktail, no green signal was detected, proving the primary antiBrUTP antibody specificity. (v) No non-specific staining due to the secondary antibodies used was detected when no primary control was performed. All experiments were performed in FM conditions to maximize rRNA synthesis. Single bar = 8 μm; double bar = 20 μm.

Similarly, when the primary antibodies were omitted, no fluorescent signal was detected (Fig. 5B v).

## Redistribution of Tip60 after RP I and II transcriptional inhibition

The *in vivo* evidence for a role of Tip60 in nucleolar RNA synthesis was further corroborated by studies involving two different transcription inhibitors. Actinomycin D (act D) inhibits RNA polymerase activity by intercalating into DNA (43). At low concentrations (0.05 μg/ml), act D selectively inhibits RP I-mediated transcription (44) and leads to nucleolar segregation, a morphological alteration generally reflecting a transient inactivation of rRNA synthesis (45). Act D treatment resulted in Tip60 accumulation into several enlarged segregated nucleoli. To further reveal the distribution pattern within these formations, we subjected our confocal image stack to Blind Deconvolution using the AutoDeblur software, in order to further improve the *z*-resolution (final *z*-axis resolution = 0.5 μm). Additionally, the signal-to-noise ratio was improved without significant loss of fluorescent signal due to photobleaching because of the reduced number of averages used. As shown in Figure 6A, Tip60 exhibits the characteristic cap-like distribution (arrows in Fig. 6A and B, green and red, respectively) previously reported for factors involved in RP I transcription, including UBF, RPA194, RPA43 and PAF53, upon act D treatment (46,47). Furthermore, there was an apparent overlap between Tip60 and UBF upon act D treatment, with Tip60 demonstrating a

more extended configuration (yellow, Fig 6B). Therefore, the redistribution of Tip60 within the segregated nucleoli in response to RP I-mediated transcriptional inhibition is consistent with Tip60 playing a role in ribosomal gene transcription, as previously reported for other factors (48,49).

The adenosine analogue 5,6-dichloro-1-β-ᴅ-ribofuranosyl-benzimidazole (DRB) is a well-known RP II-mediated transcription inhibitor (50), with the capacity to reversibly unravel nucleoli into necklace-like structures (51). Ribosomal transcripts and various transcription factors of the RP I apparatus, including UBF, reside within these structures (52). DRB induced a dramatic change in Tip60's nucleolar distribution. Specifically, Tip60 formed discrete foci, smaller than the nucleolar aggregates apparent in FM (Fig. 1A), fairly regularly distributed around the nucleolus, forming the beads of the 'necklace' (Fig. 6C, red). Tip60's distribution was unaffected by vehicle control (data not shown). A similar necklace-like pattern was observed for UBF (Fig. 6C, green). Merging the two signals revealed a high degree of overlap for all three units observed (yellow within the dotted circle; Fig 6C). Overall, the co-localization and co-behaviour of both
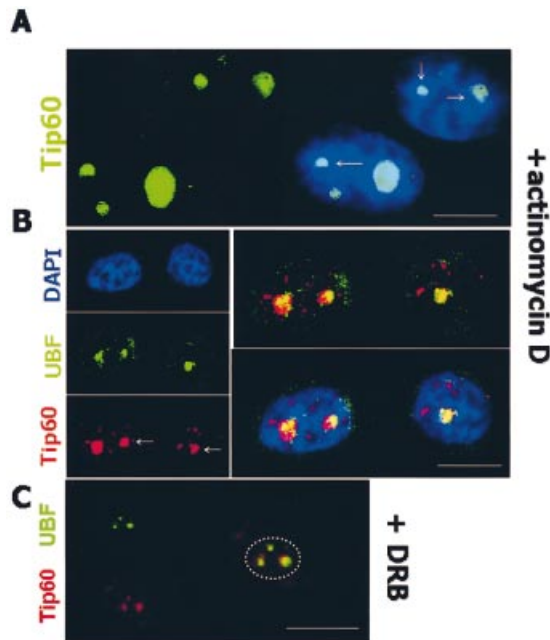
Figure 6. Tip60's distribution in response to transcriptional inhibition. (**A**) Low dose actinomycin D (0.05 µg/ml) inhibiting RP I, causes nucleolar aggregating of Tip60 (green). After applying the deconvolution algorithm, cap-like structures were also apparent (arrows), while Tip60's nucleoplasmic signal was completely abolished. (**B**) Upon act D treatment, the rDNA genes are clustered together in several highly compact nucleolar structures, as shown by UBF (green). Tip60 (red) showed a similar redistribution to UBF and the two factors co-localized in the nucleoli (yellow). The blue nuclear counterstain (DAPI) outlines the nucleolar areas. (**C**) DRB induced a nucleolar 'necklace' in the nucleolar periphery (white circle) for both UBF (green) and Tip60 (red). Bar = 10 µm.

Tip60 and UBF upon transcriptional interference (53) provide further *in vivo* evidence for the involvement of this HAT enzyme in the ribosomal transcription process.

Tip60 interacts with UBF

When human, FLAG tagged Tip60 was stably expressed in PC3M cells (Fig. 7A), and subsequently immunoprecipitated from nuclear protein extracts, one splice variant of the endogenous human UBF was specifically co-immunoprecipitated (Fig. 7B). The absence of a human antibody specific to UBF1 did not allow identification of the specific isoform complexing with Tip60. Interestingly, PAF53, the third largest RP I subunit (54), which mediates the interaction between RP I and UBF (55), was also shown to weakly interact with Tip60 (data not shown). Therefore, our data are suggestive of a potential involvement of Tip60 in the formation of the pre-initiation complex at the rDNA promoter alongside the RP I holoenzyme and UBF.

Tip60 acetylates UBF

After establishing that Tip60 interacts with UBF, we set out to investigate whether UBF could constitute a target of Tip60's enzymatic activity. In order to do this, an *in vitro* protein acetyltransferase assay was performed. We selected UBF1, by far the most potent of the two spliceforms in transcriptional activation from the ribosomal promoter (36,56), to study whether it represents a substrate for Tip60.
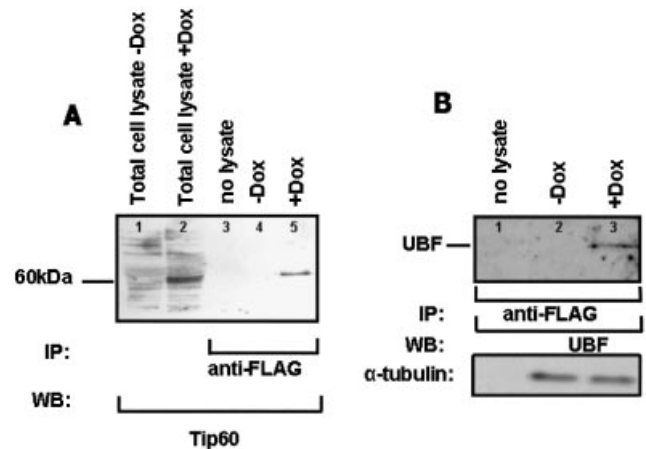
Figure 7. Tip60 is found in a complex with UBF. (**A**) Generation of Tip60α-FLAG tagged PC3M, doxycycline inducible cell line. Lanes 1 and 2 show western blots of total cell lysates in the absence or presence of doxycycline. In the latter case, a 60 kDa band corresponding to Tip60 is detected. Lanes 3–5 show immunoprecipitation experiments with the anti-FLAG antibody of nuclear extracts, in the absence (lane 4) or presence (lane 5) of induction. Lane 3 shows the specificity of the assay and serves as a negative control. Tip60 protein was only detected in the induced sample (lane 5). (**B**) Endogenous human UBF co-immunoprecipitates with Tip60. Upper panel: Tip60 was immunoprecipitated from nuclear extracts of the PC3M Tip60α inducible cell line using the anti-FLAG antibody. UBF was visualized on western blot using an anti-UBF antibody. Only one UBF spliceform is detected in lane 3. Lower panel: Alpha-tubulin loading control.

Tip60 and UBF1 were expressed in baculovirus infected Sf9 cells and purified to near homogeneity (17). PCAF, a well-studied HAT enzyme (4), was also included in the assays (data not shown). UBF1 was incubated with equal amounts of HAT enzymes and acetyl-CoA, and the acetylated product was detected using an antiAc-Lys antibody. As shown in Figure 8A, Tip60 acetylated UBF1. Interestingly, UBF1 overexpressed in insect cells, showed a low level of acetylation, consistent with the fact that post-transcriptional modification pathways such as acetylation, are active in insect cells. Both HAT enzymes used in the assay (i.e. PCAF and Tip60) were autoacetylated as previously reported (57) and (58), respectively, while UBF1 was not acetylated by PCAF (data not shown). When the Tip60 HAT-deficient mutant (Tip60$^{Q377E/G380E}$) was used in the same assay, no acetylation, of either UBF or Tip60 itself, was detected (data not shown), which further demonstrates that Tip60's HAT activity is responsible for the acetylation observed. The lower panel in Figure 8A demonstrates equal UBF loading in the different incubations. Studies are under way to examine whether UBF2 is also subjected to acetylation by Tip60.

In addition, crude histones were used as a positive control. As shown in Figure 8B, histone H4 was the major acetylation product for Tip60. Both histones H3 and H2A were also acetylated, but no acetylation of histone H2B was evident. The above substrate specificity has been previously demonstrated for Tip60 (59) and further demonstrates the functionality of our assay as well as the specific enzymatic activity of the recombinant Tip60 used.
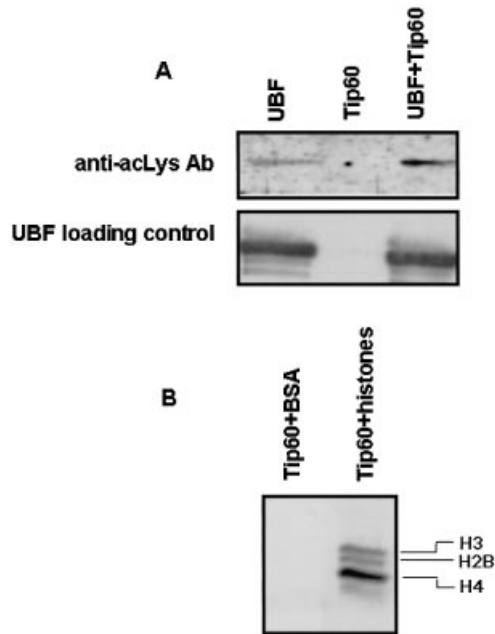
Figure 8. (**A**) *In vitro* acetylation assay demonstrating that UBF is a substrate for Tip60's acetyltransferase activity. Upper panel: The first lane shows background levels of overexpressed UBF acetylation in insect cells (see text), in the absence of Tip60, detected using an anti-acLys antibody. Tip60 in the absence of UBF shows no substrate acetylation (lane 2). In the presence of Tip60 and UBF (lane 3), there is an increase in UBF acetylation levels above background (lane 1). Lower panel: UBF equal loading control for the different incubation conditions. (**B**) Histone acetylation by recombinant Tip60. Fluorographic analysis of acetylation reactions containing recombinant Tip60 in the presence of BSA (lane 1) or core histones (lane 2), following incubation with 3H acetyl CoA.

Figure 9. (**A**) Schematic representation of the PCR primers (F and R) and the human rDNA promoter region amplified containing two *cis*-acting elements essential for high levels of transcription *in vivo*, the upstream control element (UCE) and the core element (CORE). (**B**) Chromatin immunoprecipitation using antibodies directed against Tip60 and UBF, as indicated, after 0 and 15 min treatment of LNCaP cells maintained in SDM, with 10 nM R1881. As demonstrated, both Tip60 and UBF are recruited to the rDNA promoter at the 15 min time-point (first and second panels, respectively). In addition, the histone acetylation levels also increase at the 15 min time-point (third panel), suggesting active transcription. The bottom panel represents equal loading control.

Tip60 is recruited to the rDNA promoter

After establishing that UBF is indeed a substrate for Tip60, we investigated whether Tip60 was recruited to the rDNA promoter in order to exert its effect upon local chromatin as a HAT enzyme. Ribosomal genes have been shown to be androgen responsive (60). We hypothesized that Tip60 is recruited to the human rDNA promoter upon enhancement of ribosomal gene transcription by androgens. Chromatin immunoprecipitation (ChIP) assays were performed on androgen responsive LNCaP cells using anti-Tip60 and anti-UBF antibodies, in response to androgen deprivation or synthetic androgen (10 nM R1881) (Fig. 9). The *cis*-acting elements within the human rDNA promoter, essential for high levels of transcription *in vivo*, consist of the CORE element and UCE (61) along with the transcription initiation site (62). To study Tip60's recruitment to the ribosomal gene promoter, primers flanking this region, including the UBF1 binding site, were designed (Fig. 9A), and the corresponding DNA fragment amplified by PCR and detected by autoradiography. As shown in Figure 9B, treatment with androgens resulted in Tip60's recruitment to the rDNA promoter, as early as 15 min after initiation of treatment (first panel). UBF similarly showed an early recruitment pattern, in response to androgens (Fig. 9B, second panel). In addition, the acetylation levels of H4, the Tip60-complex substrate (63), also showed an increase after 15 min of treatment with androgens (third

panel), reflecting conformational changes of the 3D chromatin structure directly related to active transcription. The bottom panel demonstrates equal loading. As a negative control, unrelated, non-specific immunoglobulin G (IgG) was used and no recruitment was shown, confirming the specificity of the antibodies used (data not shown). Our data implicate Tip60 in rRNA synthesis by demonstrating its recruitment, along with UBF, onto the human ribosomal gene promoter, upon androgen treatment, as well as the accompanying chromatin hyperacetylation.

DISCUSSION

RP I-mediated transcription and the need for chromatin modifications

RP I-mediated transcription takes place in the nucleoli of eukaryotic cells and is acutely regulated in response to a variety of physiological or pathological stimuli (38,64,65). Additionally, ribosomal gene transcription is regulated by the cell cycle (66) and tumour suppressor genes, including p53 (67). In contrast to genes transcribed by RP II and III, the molecular basis of transcription by RP I on chromatin templates has not been fully elucidated. However, the need for chromatin modification and, hence, the importance of HATs and HDACs in RP I transcriptional regulation, is beginning to emerge. Indeed, both promoter and coding regions of the rDNA gene cluster are associated with euchromatic and heterochromatic gene markers (i.e. acetylated H4 K9 and methylated H3 K9, respectively) (68). Furthermore, disruption of chromatin structure on transcriptional regulatory elements is a prerequisite for ribosomal

transcription in yeast and is probably mediated by specific DNA–protein interactions occurring prior to transcription (69). Similarly, in mammalian cells, chromatin remodelling has been demonstrated to constitute an essential step preceding transcription initiation (70).

The recruitment of the human ribosomal transcription factor UBF to the RP I promoter activates transcription, allowing recruitment of the SL-1 complex and the RP I holoenzyme (71). UBF, a member of the high mobility group (HMG)-box proteins (72), forms dimers that loop approximately 140 bp of rDNA into a single turn producing a transcriptionally active structure termed 'enhancesome' (6). The switch between the inactive nucleosomal and the active enhancesomal state could provide the basis for gene activation and control the number of active ribosomal genes. This transition may be regulated by recruitment of HAT activity, as suggested (8,9). Our objective was to examine the potential involvement of Tip60, a HAT with clear nucleolar localization, in ribosomal gene transcription.

Tip60's involvement in ribosomal gene transcription: the evidence

We have shown that Tip60 resides in the nucleolus of a range of CaP cell lines. Furthermore, we demonstrated the exclusion of CBP from the nucleolus of these cell lines, proposing a potential cell type specific nuclear distribution profile of HATs. Based on the promiscuity that characterizes HAT enzymes, we hypothesized that Tip60 acts as one of the main providers of acetyltransferase activity in LNCaP cells, an androgen-dependent, AR expressing prostate cancer cell line. Confocal immunofluorescence co-localization studies along with electron immunogold labelling further supported our hypothesis and indirectly implicated Tip60 in ribosomal gene transcription.

Ribosomal inhibition and *in vivo* run-on transcription assays provided functional evidence of such an involvement. Evidence for a physical interaction between Tip60 and a ribosomal specific transcription factor, UBF, was provided by immunoprecipitation assays and *in vitro* acetylation assays revealed weak but reproducible and detectable above baseline acetylation of UBF1 by Tip60. Acetylation of specific UBF lysine residues may result in a small, overall, degree of acetylation detected by means of conventional antibodies, but important conformational/functional changes at the molecular level. It would be of interest to investigate whether TAF$_I$68, a subunit of the basal rDNA transcription factor SL1 (selectivity factor 1), recently shown to be acetylated by PCAF (73), also constitutes a substrate for Tip60.

Previous reports have linked Esa1, the yeast homologue of Tip60, with enhanced transcription from specific ribosomal protein promoters (74). Interestingly, microarray experiments performed with cells over-expressing Tip60 have shown a 4- to 6-fold increase in the human 18S and 28S ribosomal genes, in addition to several ribosomal genes (manuscript in preparation), supporting our results implicating Tip60 in ribosomal gene transcription. Using ChIP experiments, we established Tip60's role as a HAT in the ribosomal transcription process, in addition to its FAT activity towards UBF [reviewed in (4)].

Tip60 was recruited to the human ribosomal gene promoter, along with UBF, upon androgen stimulation, in the androgen-dependent prostate cancer cell line, LNCaP. Interestingly,

60 min after the beginning of the treatment, Tip60 no longer occupied the promoter (data not shown), suggesting an 'on/off' recruitment pattern similar to that reported for the androgen responsive PSA gene (17). Furthermore, we demonstrated an increase in H4 acetylation levels reflecting a chromatin transition to a transcriptionally active state. That ribosomal transcription in mammals is regulated in response to growth and differentiation stimuli is well established, however, the molecular mechanism underlying this regulation is not yet fully clarified. Hormones, including androgens (60), and growth factors enhance ribosomal transcription (38,75), whilst UBF overexpression induces cell proliferation in a variety of cellular systems (76,77). Experiments are under way in order to establish whether Tip60 is recruited exclusively to the promoter region or tracks along the gene coding region together with the RP I holoenzyme. In addition to implicating Tip60 in ribosomal chromatin modifications, our data propose a potential mechanism based on which androgens may, at least in part, exert their stimulating effect on ribosomal transcription via the recruitment of Tip60, a HAT with a well-established AR co-activator function (15,17).

UBF1 or UBF2?

We were intrigued to detect only one form of UBF rather than the typical UBF1/UBF2 heterodimer in the Tip60 immuno-precipitate, especially since the anti-UBF antibody used is capable of identifying both isoforms. While our data are not conclusive as to which of the two UBF spliceforms complexes with Tip60, further studies are currently under way to examine whether UBF2 is also an acetylation substrate for Tip60. UBF1 is considered to represent the most important of the two spliceforms in ribosomal transcription, and it would be tempting to speculate that it is UBF1 that complexes with Tip60, since it is also acetylated by Tip60.

Indeed, while HMG boxes 1 and 2 are the main acetylation sites within UBF1 (8), UBF2 lacks 37 amino acids from HMG box 2 as result of alternative splicing (78) and was found to be functionally inactive in promoter function (79,80). Similarly, in a more recent report, the natural UBF2 splice variant exhibited a severely impaired capacity to activate RPI transcription (56). Interestingly, the UBF1 variant predominates in rapidly growing cells (81). However, the role of UBF2 should not be underestimated, nor its potential involvement in ribosomal transcription. Interestingly, UBF2 has been recently identified as an RP II transcriptional enhancer (82), and, hence, implicated in mRNA synthesis, a process in which Tip60 plays an essential role as a nuclear receptor co-activator and a HAT (16).

Model for Tip60's function in rDNA transcription

In this study, we have demonstrated the involvement of Tip60 protein in human ribosomal gene transcription. Although the precise molecular basis of this involvement is not fully elucidated, there are several mechanisms via which Tip60 could modulate ribosomal transcription: (i) Tip60's recruitment to UBF could activate transcription by the acetylation of UBF, as previously shown for CBP (8); (ii) in addition to its FAT activity, Tip60 could stimulate rDNA transcription through histone acetylation and alteration of the chromatin structure, either as part of the UBF complex or via its direct recruitment to the rDNA gene promoter where it could exert

its HAT activity; (iii) Tip60 may modulate ribosomal transcription through its direct interaction with HDAC1, an established repressor of ribosomal transcription (8,83). Indeed, the AR, Tip60 and HDAC1 form a trimeric complex upon the endogenous androgen-responsive PSA promoter, thus modulating AR's transcriptional activity via acetylation and deacetylation (17). Unfortunately, we have, at present, no direct evidence as to the causal involvement of Tip60 in rDNA transcription activation and the underlying molecular mechanism. However, Tip60's recruitment profile to the rDNA promoter suggests a multi-step process, where HAT activity is recruited early upon transcription stimulation, possibly to allow recruitment of chromatin remodelling complexes, and, thus, permit the formation of the transcription initiation complex. Interestingly, the higher degree of co-localization between Tip60 and UBF for cycling cells compared to G_0 arrested cells, also supports the enhancing role of Tip60 in ribosomal transcription. Therefore, our data put forward a model in which Tip60 has a dual role to play in ribosome gene regulation, both via interacting and directly acetylating UBF and through its recruitment to the human rDNA gene promoter.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

ACKNOWLEDGEMENTS

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