

# Human Arf tumor suppressor specifically interacts with chromatin containing the promoter of rRNA genes

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**The tumor suppressor Arf (Alternative Reading Frame) protein (p14<sup>ARF</sup> in human and p19<sup>ARF</sup> in mouse) is mainly located in the nucleolus consistent with its subcellular localization, the protein has been shown to specifically interact with 5.8S rRNA and with B23/Nucleophosmin and to regulate ribosome biogenesis. Here, we show that the p14<sup>ARF</sup> protein interacts with chromatin and is recovered by chromatin immunoprecipitation (ChIP) in a fraction that contains a DNA sequence of the rRNA gene promoter. In addition, topoisomerase I (Topo I) that has been shown to interact with p14<sup>ARF</sup> coprecipitates with p14<sup>ARF</sup> containing chromatin. These data, in view of the function for Topo I in rRNA transcription, are consistent with a role for the p14<sup>ARF</sup>-Topo I complex in rRNA transcription and/or maturation.**

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## Introduction

The Arf protein (p14<sup>ARF</sup> in man and p19<sup>ARF</sup> in mouse) is one of the two tumor suppressor proteins encoded by the p16<sup>INK4a</sup>/CDKN2 gene located on chromosome 9p21 in man (for a review see Lowe and Sherr, 2003). Whereas p16<sup>INK4a</sup> protein (encoded by the  $\alpha$  transcript) acts as an inhibitor of cyclin D-CDK 4-6 (Cyclin Dependant Kinase), p14<sup>ARF</sup> and p19<sup>ARF</sup> (encoded by the alternative  $\beta$  transcript) have been shown to stop proliferating cells in G<sub>1</sub> and also G<sub>2</sub> phases (Quelle *et al.*, 1995). A number of experiments with the murine and human species have shown that this cell cycle inhibitory function results from sequestration of Mdm2, a protein involved in p53 degradation through ubiquitination and translocation to the proteasome, in the nucleolus (Pomerantz *et al.*, 1998; Zhang *et al.*, 1998; Honda and Yasuda, 1999). Accordingly, nucleoplasmic p53 is stabilized and can exert its antiproliferative and proapoptotic effects. More recent studies have shown that Arf protein can activate p53 without relocating

Mdm2 in the nucleolus (Llanos *et al.*, 2001; Korgaonkar *et al.*, 2002). In this respect, nucleolar Arf location does not make sense and suggests that it interferes with some functions taking place in this organite. Furthermore, a number of evidence have clearly shown that Arf can stop proliferation in a p53-defective context. For example, p19<sup>ARF</sup> inhibits cell cycle progression of mouse embryonic fibroblasts (MEFs) invalidated for p53, Mdm2 or both (Carnero *et al.*, 2000; Weber *et al.*, 2000). Also p14<sup>ARF</sup> has been reported to inhibit E2F1 expression in p53<sup>-/-</sup> human cells (Eymin *et al.*, 2001). Finally, always in a p53<sup>-/-</sup> context, the inhibition of *in vitro* and *in vivo* cell proliferation mediated by Arf has been shown to require a transient G<sub>2</sub> arrest followed by apoptosis (Eymin *et al.*, 2003).

Several partners of Arf have been recently characterized but no functional evidence has been so far reported. We have identified topoisomerase I (Topo I) as one of the partners of p14<sup>ARF</sup> and shown that the interaction of the two proteins stimulates the relaxation activity of Topo I *in vitro* (Karayan *et al.*, 2001; Ayrault *et al.*, 2003). While the functional significance of this complex is currently unknown, its nucleolar localization suggests that it might be implicated in some cell events taking place in this organite. In this respect, p19<sup>ARF</sup> has been recently shown to modulate rRNA processing (Sugimoto *et al.*, 2003). In addition, p14<sup>ARF</sup> protein has been shown to induce degradation of the nucleolar protein B23, resulting in block of preribosomal RNA and cell death induction (Itahana *et al.*, 2003). These data suggest that the inhibitory activity of Arf on cell proliferation is mediated through its regulatory effects on ribosome biogenesis.

In this report, we show that the Topo I-p14<sup>ARF</sup> complex found in human cells is present in chromatin. In addition, by using a ChIP approach, we have found that p14<sup>ARF</sup> can specifically associate with a chromatin fraction that contains the rRNA gene promoter. These data argue in favor of a role for p14<sup>ARF</sup> in modulating rRNA transcription.

## Results

*Relaxation of supercoiled DNA by Topo I is facilitated by preincubation of the substrate with p14<sup>ARF</sup>*

We recently described an *in vitro* and *in vivo* functional interaction between the human tumor suppressor

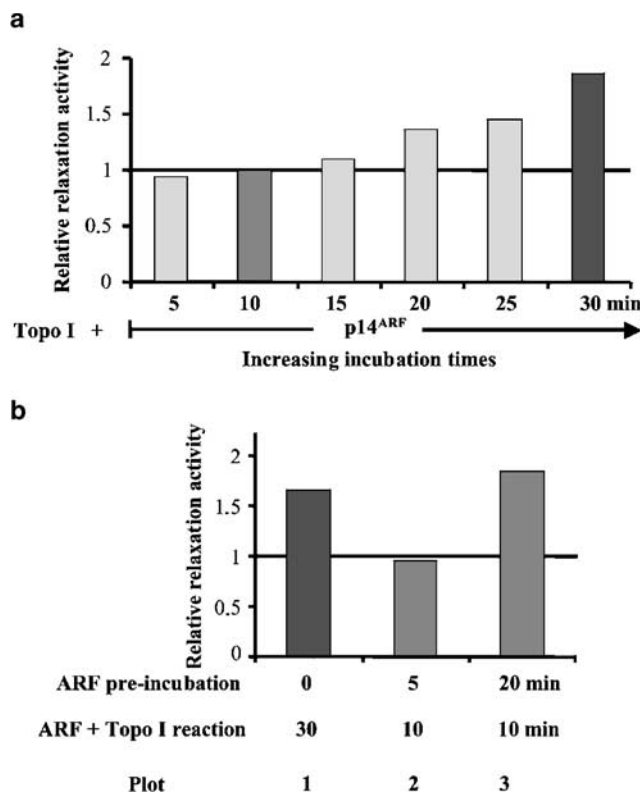
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protein p14<sup>ARF</sup> and human Topo I (Karayan *et al.*, 2001; Ayrault *et al.*, 2003). Briefly, *in vitro* incubation of p14<sup>ARF</sup> and Topo I with supercoiled plasmid DNA was shown to increase the relaxation levels of the DNA substrate by comparison with relaxation levels measured in the presence of Topo I alone. Two alternative possibilities were considered to explain the mechanism of the stimulatory activity of p14<sup>ARF</sup> on Topo I. At first, we reasoned that binding of p14<sup>ARF</sup> to Topo I might increase the affinity of the enzyme toward its substrate resulting in more efficient relaxation activity. Alternatively, in view of the unusual basic properties of p14<sup>ARF</sup> (pI > 12.4), we speculated that the protein might directly interact with plasmid DNA in such a way as to facilitate DNA binding to Topo I.

In order to distinguish between these two explanations, we utilized the *in vitro* assay previously used for measuring Topo I relaxation activity. Shortly, protein extracts were prepared by lysis of SF9 cells infected with p14<sup>ARF</sup> or Topo I recombinant baculoviruses (Ayrault *et al.*, 2003). Extracts of SF9 cells infected with wild-type baculovirus are used as control (AcNPV). Preliminary assays were performed in order to determine the minimal amounts of each protein extract that did not induce any detectable relaxation of plasmid DNA upon incubation for 30 min at 37°C. These conditions were selected for all reconstitution experiments to be described thereafter.

In a first series of assays, Topo I plus AcNPV or Topo I plus p14<sup>ARF</sup> proteins were incubated together in the presence of plasmid DNA during different increasing times from 5 to 30 min. Upon running samples on agarose gels and scanning of the gels, relaxation activity was estimated by comparing the activity of p14<sup>ARF</sup> + Topo I to that of AcNPV (wild baculovirus) + Topo I (Figure 1a). As expected from our previous results, relaxation activity increased with times of reaction. Since Topo I alone did not exhibit any activity under these experimental conditions, this increase is attributed to the presence of p14<sup>ARF</sup> in the extracts. The kinetics of the process indicated that this increase was delayed for the first 15 min, then raised during the next 15 min, suggesting that p14<sup>ARF</sup> had to interact with the enzyme or its substrate before exerting its stimulatory activity.

In a second set of experiments, plasmid DNA was incubated with p14<sup>ARF</sup> alone for 5 and 20 min, then Topo I was added to the mixture and incubation was continued for 10 min more (Figure 1b), and note that the reaction conditions are to be compared to those of the second plot in Figure 1a, as in both cases the incubation in the presence of the two proteins was going on for 10 min). After 5 min of preincubation of p14<sup>ARF</sup> with plasmid DNA plus 10 min in the presence of Topo I (plot 2 Figure 1b), there was no noticeable difference when compared to the two first plots in Figure 1a. On the opposite, after 20 min preincubation, the relaxation levels reached at least those observed after the incubation of both proteins for 30 min (compare plot 1 to plot 3, Figure 1b). In these experimental conditions, Topo I appears to be three-fold more efficient. Our interpretation of these data is that p14<sup>ARF</sup> exerts its *in vitro*



**Figure 1** p14<sup>ARF</sup> facilitates Topo I induced relaxation on supercoiled DNA. Sf9 cells were separately infected with AcNPV, BacHARFwt or BacHTopo I wt. BacHARF wt or AcNPV extracts were mixed with 10 µg of BacHTopo I wt cells extracts, and diluted before assaying Topo I activity. The first dilution that did not exhibit any relaxation activity was used in all the experiments. After reactions, the samples were run on 1% agarose gel and stained with ethidium bromide before scanning for quantification of the stimulating effect (for more details, see Ayrault *et al.*, 2003). (a) Supercoiled plasmid was incubated at 37°C with Topo I (30 min for all samples) and p14<sup>ARF</sup> or AcNPV were added for different times indicated on the X-axis. Briefly, the DNA relaxation activity is expressed as the ratio of p14<sup>ARF</sup> + Topo I/AcNPV + Topo I. The baseline (level 1 on the Y-axis) corresponds to the absence of Topo I stimulation by p14<sup>ARF</sup>. After 10 min latency, activity increases in function of the time of reaction. (b) The relaxation activity of Topo I was measured and expressed like in a. Plot 1: Coincubation of p14<sup>ARF</sup> + Topo I and AcNPV + Topo I during 30 min at 37°C, internal control. Plots 2 and 3: p14<sup>ARF</sup> or AcNPV were preincubated for 5 or 20 min with the supercoiled plasmid DNA substrate before addition of Topo I for 10 min at 37°C

stimulating effect via a direct interaction with the supercoiled plasmid.

While these data argued in favor of a direct interaction of p14<sup>ARF</sup> with DNA, they obviously did not mimic *in vivo* conditions, as the structural complexity of chromatin was not considered in these assays.

#### p14<sup>ARF</sup> is found in protein fraction associated with chromatin

To detect a possible interaction between p14<sup>ARF</sup> and chromatin, we first performed protein subfractionation experiments according to the procedure published by Gladden and Diehl with a few modifications (see

Materials and methods), on two tumor cell lines, H-358 and 293, that endogenously express p14<sup>ARF</sup>. Cellular proteins were separated in four fractions: soluble proteins were removed first by Triton X-100. DNase I digestion, followed by extraction with ammonium sulfate, then released chromatin-associated proteins referred to as XAP. Upon washes of the cell extracts with 2 M NaCl, the remaining proteins corresponded to the insoluble structural nuclear matrix proteins and nuclear matrix-associated proteins, referred to as the insoluble fraction (Burke *et al.*, 2001; Gladden and Diehl, 2003). Supernatants from each extraction step and the insoluble fraction were quantified by BCA kit (Bicinchoninic Acid, SIGMA). The same amounts of total proteins from each fraction were analysed by SDS-PAGE and Western blotting. As shown in Figure 2, a large part of p14<sup>ARF</sup> was found in the chromatin-associated proteins fraction (XAP, lane 2). However, p14<sup>ARF</sup> was also detected in the nuclear matrix and the soluble fractions. The presence of the protein in the soluble fraction was expected and corresponds to both cytoplasmic Arf and soluble nuclear Arf. To validate our results, H-358 cell extracts were analysed in parallel for the distribution of a soluble (Rho) and a DNA binding protein (Topo I) in the different fractions (Figure 2). As expected, the Rho protein used as soluble

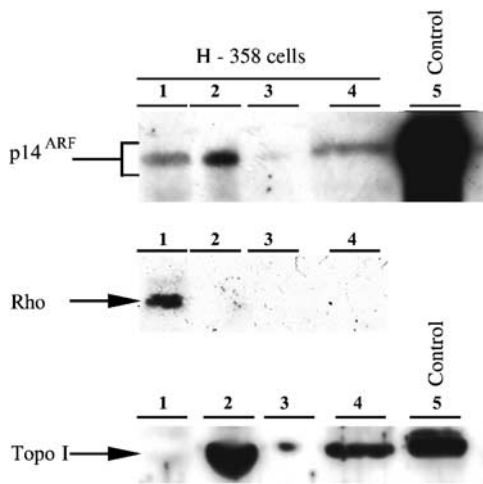
control, was only detected in the soluble fraction (Figure 2, second blot, lane 1), whereas Topo I was present in the XAP as well as in the nuclear matrix-associated protein fractions (Figure 2, third blot, lanes 2 and 4). The same results are observed with 293 cells (data not shown).

These experiments indicate that p14<sup>ARF</sup> is released after DNase I digestion, suggesting that this protein belongs to chromatin or chromatin-associated proteins.

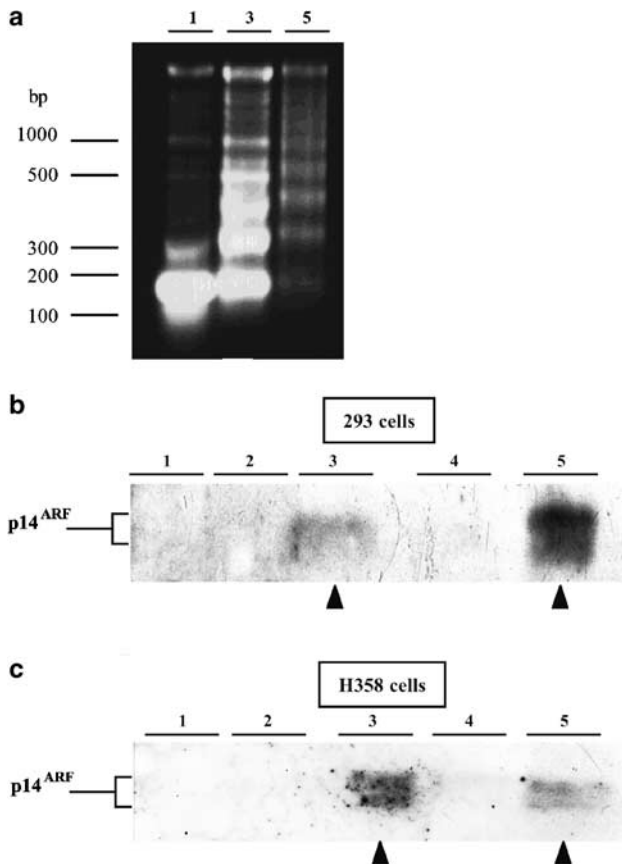
*Part of cellular p14<sup>ARF</sup> cosediments with a particular chromatin fraction*

The fact that p14<sup>ARF</sup> is a very basic protein that may nonspecifically interact with different molecules including DNA could readily explain its association with various proteins and protein structures, including Topo I and chromatin. In that case, it should be undifferently associated with any soluble or insoluble chromatin fractions, whatever its active or inactive status. On the opposite, if a specific interaction exists, the protein is likely to interact with particular sets of chromatin. To address this point, we carried out experiments to distinguish the so-called active and inactive chromatin parts, before separating them by sucrose-gradient fractionation, according to the protocol published by Arnan *et al.* (2003) with minor modifications. This method, consisting of a mild digestion of nuclei by the micrococcal nuclease, relies on biochemical studies showing that transcriptionally active chromatin exhibits increased sensibility to nucleases (Rose and Garrard, 1984; Reyes *et al.*, 1997). Nuclei were isolated from H-358 or 293 cells. The contaminant soluble fraction of Arf was eliminated by sequential washes, then the nuclei were submitted to a mild digestion with micrococcal nuclease (3 U for 5 min at room temperature) to induce internucleosomal cleavage. In order to separate the subpopulation of chromatin generated by nuclease activity, the resulting extracts were centrifuged through a linear density 5–30% sucrose gradient and five fractions were collected, designated 1–5 from the top to the bottom of the tube and analysed by Western blotting and for their DNA content (Figure 3). Experiments were carried out three times for each cell line and results were perfectly reproducible.

Fraction 5 (Figure 3a) is a complex mixture of large DNA fragments from nondigested chromatin. These fragments come from undisrupted and nuclear debris and also from insoluble, micrococcal nonsensitive, chromatin (compare lane 5 with lane 1 that contains mononucleosomal digestion fragments from fraction one; see also Reyes *et al.*, 1997). As expected, Western blotting analysis revealed the presence of p14<sup>ARF</sup> in fraction 5 including the protein trapped in the undamaged nuclei and the protein associated with insoluble chromatin bound to the nuclear scaffold after fractionation. p14<sup>ARF</sup> was also detected in fraction 3 in both cell lines studied, suggesting the existence of an interaction with a particular population of chromatin comprised of essentially mono to trinucleosomes. By assuming the idea that this chromatin was sensitive to the nuclease, we



**Figure 2** p14<sup>ARF</sup> cosediments with chromatin-associated proteins. H-358 cells ( $1 \times 10^7$  cells) were first treated with Triton X-100 in order to get rid of soluble proteins (soluble fraction lane 1). Upon centrifugation, cells were treated with DNase-I in order to release chromatin-associated proteins (lane 2). After washes with 2 M NaCl (lane 3), resulting pellets constituted the nuclear matrix protein fraction (lane 4). A measure of 30  $\mu$ g of total proteins from each fraction were loaded on 14% acrylamide gel for Western blotting analysis. p14<sup>ARF</sup> was detected with a polyclonal antibody (C-18 Santa Cruz) and visualized by enhanced chemiluminescence (ECL, Amersham). Positive controls (lane 5) BacHARFwt protein extracts for detection of p14<sup>ARF</sup>, and BacHTopoI for detection of Topo I. In order to validate the fractionation, protein extracts from H-358 cells were separated on 14% polyacrylamide gel, transferred to immobilon membranes that were incubated with a monoclonal antibody against the soluble Rho protein (kindly provided by Dr N Bourmeyster). The same procedure was used to detect the DNA binding protein Topo I with a rabbit polyclonal antibody (Topogen)



**Figure 3** Active chromatin is enriched in p14<sup>ARF</sup>. Nuclei were isolated from 293 and H-358 cells and were digested with 3 U of micrococcal nuclease for 5 min at room temperature. Resulting digests were loaded onto a linear 5–30% sucrose gradient and ultracentrifuged at 20 000 r.p.m. (Beckman, rotor SW-50) for 18 h at 4°C. Five fractions were collected from the top (tube 1) to the bottom (tube 5) of the gradient. Every fraction was subdivided into two samples, one for DNA analysis on 1% agarose gel (**a**), the other for p14<sup>ARF</sup> detection by SDS-PAGE and immunoblotting with C-18 anti p14<sup>ARF</sup> antibodies (**b** and **c**)

speculate that p14<sup>ARF</sup> might interact with a particular set of active chromatin (Rose and Garrard, 1984).

These results accredit the existence of a direct interaction between p14<sup>ARF</sup> and DNA. The latter experiments comfort the existence of an *in vivo* interaction with a particular chromatin fraction. Regarding this point, previous biochemical studies have demonstrated that only a fraction of actively transcribed chromatin is easily solubilized following nuclease treatment of isolated nuclei, while the remaining portion is tightly bound to the nuclear matrix (Kamakaka and Thomas, 1990).

#### *Arf* interacts with chromatin in association with *Topo I*

The results demonstrated by these fractionation experiments indicate that p14<sup>ARF</sup> is found associated with a particular fraction of active chromatin. In that context, and given the fact that *Topo I* was detected in both chromatin and nuclear matrix fractions (Figure 2, third

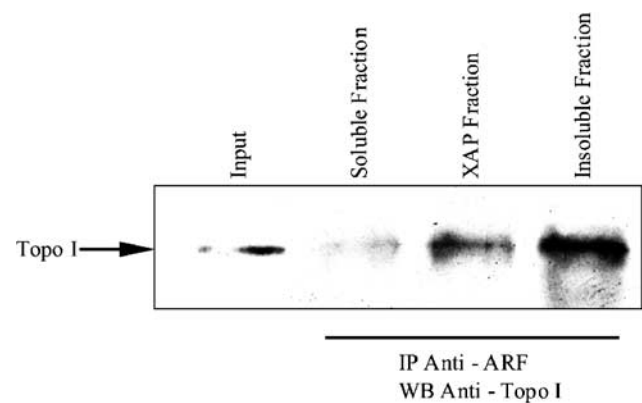
blot, lanes 2 and 4), we focused on the localization of the *Arf*-*Topo I* complex.

In order to assign the p14<sup>ARF</sup>/*Topo I* complex to one of these fractions, immunoprecipitation experiments with anti-p14<sup>ARF</sup> antibodies, followed by immunoblot with anti-*Topo I* antibodies were carried out. As shown in Figure 4, a signal was detected in chromatin as well as in insoluble fractions showing that a p14<sup>ARF</sup>/*Topo I* complex was present in chromatin fraction and nuclear matrix fraction. In contrast, the signal was weaker in the soluble protein fraction. As control, immunoprecipitation experiments with anti-p14<sup>ARF</sup> 73SA serum, followed by p14<sup>ARF</sup> detection with C18 (Santa Cruz) were performed indicating the presence of p14<sup>ARF</sup> in all fractions (data not shown).

To summarize, these data indicate that the soluble part of p14<sup>ARF</sup> protein is not associated with *Topo I* and that a p14<sup>ARF</sup>/*Topo I* complex is essentially associated with the chromatin fraction and the nuclear matrix. Of note, nuclear fractionation studies have previously shown that part of transcriptionally active DNA, including ribosomal DNA genes, is tightly associated with the nuclear skeleton or nuclear matrix while inactive loci are not (Jackson and Cook, 1985; Smith and Rothblum, 1987; Jackson *et al.*, 1993).

#### *Chromatin immunoprecipitation (ChIP) assay demonstrates association of p14ARF specifically with human rRNA gene promoter*

We have previously reported that most (if not all) of the p14<sup>ARF</sup>/*Topo I* complex is located in the nucleolus (Karayan *et al.*, 2001). For this reason, and because of the requirement for *Topo I* in accurate transcription of supercoiled rDNA (Garg *et al.*, 1987; Zhang *et al.*,



**Figure 4** p14<sup>ARF</sup>-*Topo I* complex is localized in chromatin and nuclear matrix protein fractions. Protein extracts from H-358 cells were fractionated into soluble, chromatin and insoluble fractions as described (Burke *et al.*, 2001; Gladden and Diehl, 2003). Soluble fraction, chromatin fraction (XAP) and insoluble fraction were immunoprecipitated with rabbit polyclonal 73SA anti-*Arf* serum. Immune complexes were revealed with an antibody against human *Topo I* (Topogen) or against p14<sup>ARF</sup> (data not shown) (C-18, Santa Cruz). As expected, anti-*Topo I* antibodies detected a band running at a 100 kDa position in the input (lysate). XAP represents chromatin-associated proteins. WB: Western blot. IP: immunoprecipitation

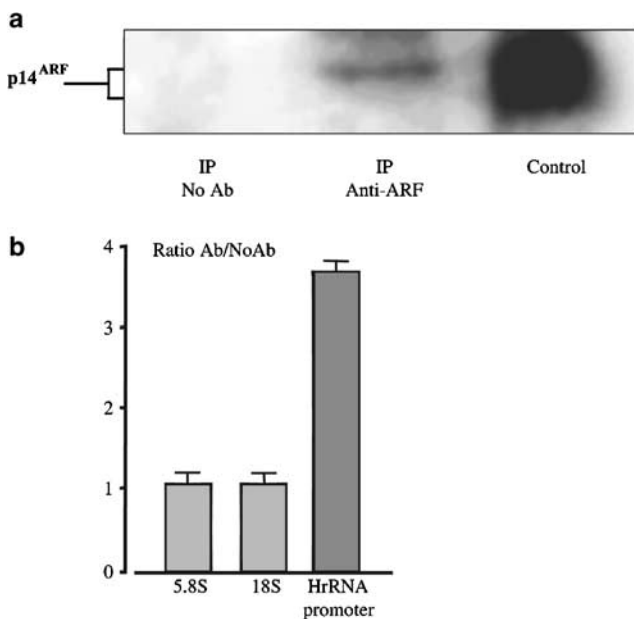
1988), we addressed the possibility that p14<sup>ARF</sup> was able to bind the rRNA promoter gene by carrying chromatin immunoprecipitation experiments (ChIP) on 293 cells. At first, direct Western blot analysis was performed on lysates prepared from cells previously cross-linked and sonicated as described, then immunoprecipitated with the anti-p14<sup>ARF</sup> 73SA serum. The protein was repeatedly detected in the immunoprecipitated extracts but not in the control samples without antibody, thus assessing the quality of our antibody for ChIP experiments (Figure 5a). Next, DNA isolated from immune complexes was amplified with different primers. Those aimed at amplifying the rDNA promoter represented sequence -126 and +20, the transcription start site being taken as the zero reference. Four different genomic sequences, namely GAPDH, 5S, 5.8S and 18S were amplified in parallel. The latter 18S, 5.8S and 5S sequences were selected as internal controls as they are close to the promoter. Semiquantitative PCR on several independent experiments was performed in parallel on chromatin samples before (referred to as the input) and after immunoprecipitation (see Materials and methods for semiquantitative PCR protocol). The PCR signals for the controls were found to be equivalent in

nonimmunoprecipitated chromatin and immunoprecipitated chromatin. In contrast, the intensity of the PCR signal for the chromatin samples containing the rDNA promoter, was about four-fold higher in the immunoprecipitated chromatin than in the nonimmunoprecipitated one (Figure 5b). Identical results were obtained with a second set of primers amplifying the promoter sequence -49 to +4 (data not shown). These data are consistent with a direct and specific interaction between p14<sup>ARF</sup> and the rDNA promoter.

## Discussion

The data reported herein indicate the existence of a specific interaction between the nucleolar p14<sup>ARF</sup> protein and chromatin. Several pieces of evidence consistent with it, are summarized thereafter. First, we have shown that p14<sup>ARF</sup> was strongly present among proteins bound to chromatin and proteins associated to nuclear matrix. As expected, a similar pattern was observed for Topo I. Coimmunoprecipitation experiments have clearly demonstrated that the ARF-Topo I complex previously described (Karayan *et al.*, 2001; Ayrault *et al.*, 2003) was recovered in these same two protein fractions, particularly in the insoluble one. These results led us to investigate the opportunity for p14<sup>ARF</sup> to interact with chromatin, as we had previously shown *in vitro* that the protein alone was able to modify the structural conformation of a supercoiled DNA plasmid. We carried out chromatin fragmentation experiments, and p14<sup>ARF</sup> was found in a particular chromatin fraction that exhibited a nuclease sensitivity corresponding to an actively transcribed chromatin (Rose and Garrard, 1984; Reyes *et al.*, 1997).

As p14<sup>ARF</sup> is a nucleolar protein, and as the nucleolar chromatin associated to nuclear matrix contains the rDNA genes, we have proceeded to chromatin immunoprecipitation experiments by using anti-p14<sup>ARF</sup> antibodies. We have succeeded in precipitating a chromatin fraction whose DNA contains the promoter of the rRNA genes. The fact that immunoprecipitated chromatin was not enriched in sequences corresponding to other ribosomal rDNA segments, namely 5S, 5.8S and 18S, argues in favor of the specificity of this interaction. Also, this chromatin fraction was not enriched in a DNA segment corresponding to GAPDH gene used as a negative control. Thus, although p14<sup>ARF</sup> is assumed to be a sticky protein in view of its highly positive charge, it appears not to interact in a nonspecific way with chromatin. As a whole, these data are consistent with a functional link between p14<sup>ARF</sup> and the rDNA promoter-containing chromatin fraction. This interaction immediately raises the questions of its functional significance and the nature of the partner(s) to which p14<sup>ARF</sup> is attached in the chromatin fraction. *In vitro* data presented at the beginning of this report indicate that this might be DNA itself. For many reasons, we favor the notion that a specific binding of p14<sup>ARF</sup> to a rDNA promoter gene sequence would be mainly



**Figure 5** p14<sup>ARF</sup> links the rRNA gene promoter. **(a)** 73SA antiserum recognizes p14<sup>ARF</sup> crosslinked with chromatin after immunoprecipitation. Samples of p14<sup>ARF</sup> crosslinked to sonicated chromatin were immunoprecipitated with 73SA serum and the protein detected on Western blot with the same antibody (IP Anti-ARF). Immunoprecipitation controls were performed by immunoprecipitating p14<sup>ARF</sup> with protein A agarose beads only (IP No Ab). Control: positive control p14<sup>ARF</sup>. **(b)** Immunoprecipitated DNA was amplified by semiquantitative PCR, using 5.8S, 18S genomic primers and rRNA promoter primers. Amplified DNA was loaded onto 2.5% agarose gel and amplification levels were deduced from scanning measurement (Scan ImageBeta 4.02 software). The Y-axis corresponds to the ratio antibody/no antibody. Amplification of 5S and GAPDH sequences gave results identical to those with 5.8S and 18S primers (not shown)

dependent on the accessibility of p14<sup>ARF</sup> to this rDNA promoter sequence. This hypothesis is attractive as it conditionates the binding of p14<sup>ARF</sup> to chromatin to the conformational chromatin status.

An alternative possibility consists in the formation of the p14<sup>ARF</sup>-chromatin complex through its interaction with Topo I.

What can be the biological significance of the p14<sup>ARF</sup>-Topo I complex? Considering that Topo I is necessary for rDNA transcription (Garg *et al.*, 1987; Zhang *et al.*, 1988) and that p14<sup>ARF</sup> stimulates the cleavage activity of the enzyme *in vitro* (Karayan *et al.*, 2001; Ayrault *et al.*, 2003), we propose the following scenario: upon oncogenic stress p14<sup>ARF</sup> production is stimulated, resulting in proliferation cessation either by stabilizing p53 or independently of p53. In the latter case, a portion of p14<sup>ARF</sup> may move to the nucleolus and attach to chromatin containing rDNA promoter. In a second step, Topo I is recruited, binds p14<sup>ARF</sup>, then is stimulated for inducing nonphysiological breaks in rDNA that interfere with ribosomal RNA synthesis. This hypothesis comforts the recent notion that Topo I could be a proapoptotic endonuclease (Pourquier *et al.*, 2001).

Another explanation emerges from results recently published by two groups (Boon *et al.*, 2001; Sherr, 2001). Sherr and collaborators have reported data showing that p19<sup>ARF</sup>, the murine form of Arf, interferes with biosynthesis and maturation of rRNA in murine cells (Sugimoto *et al.*, 2003). While no clear mechanistic explanation was provided, the authors proposed that p19<sup>ARF</sup> could slow ribosome biogenesis in response to certain stress signals. p19<sup>ARF</sup> would therefore oppose Myc that has been shown to stimulate cell growth by upregulating ribosome biogenesis (Boon *et al.*, 2001; Eisenman, 2001). Also the recent finding of an interaction between p14<sup>ARF</sup> and nucleolar nucleophosmin/B23 protein that stops preribosomal RNA processing strengthens the notion that Arf exerts its antiproliferative function through interfering with ribosome biogenesis (Itahana *et al.*, 2003).

To integrate our own results to this scheme, by analogy with the role for Topo I in stimulation of SR proteins involved in splicing of messenger RNA (Rossi *et al.*, 1996), it is tempting to assume that it also might be involved in ribosomal RNA maturation.

Because the two human and murine Arf proteins exhibit several differences at the structural level whose functional relevance is not currently known, it will be important to show the presence of p19<sup>ARF</sup>-Topo I complex and also detect the presence of the p19<sup>ARF</sup>-chromatin complex containing the rDNA promoter. Alternatively, it is possible that p14<sup>ARF</sup> and p19<sup>ARF</sup> exhibit their inhibitory capacities through different mechanisms that are related to their structural differences. If we assume that a complex between p19<sup>ARF</sup> and the rDNA promoter gene also exists in mouse, it is important to underscore the existence of CpG island in the human but not in the murine promoter suggesting that the molecular control of rRNA expression may be different among the two species. In this respect, Pex19, a protein partner of p19<sup>ARF</sup> has been shown to retain

p19<sup>ARF</sup> in the cytoplasm by virtue of its interaction with the carboxyl terminus of the protein that is absent in the human p14<sup>ARF</sup> protein (Wadhwa *et al.*, 2002). This accredits the notion that p14<sup>ARF</sup> and p19<sup>ARF</sup>, although very comparable in terms of function do not use the same mechanisms to fulfill this function.

## Materials and methods

### Cell culture

Mammalian cells were grown (37°C, 5% CO<sub>2</sub>) in Dulbecco's modified Eagle medium (293 cell line) or in RPMI 1640 (H-358 cell line) supplemented with 10% fetal bovine serum and antibiotics (InVitrogen). The Sf9 subclone of *Spodoptera fugiperda* cells IPLB-SF21-AE (Vaughn *et al.*, 1977) was maintained in Grace's insect medium (InVitrogen) supplemented with Yeastolate and Lactalbumine hydrolysate at 3.3 g/l each, 10% fetal bovine serum-insect qualified and Penicillin 5000 UI + Streptomycin 5000 µg (InVitrogen).

### Topoisomerase I stimulation assay

SF9 cells were infected with ACNPVwt (*Autographia californica* nuclear polyhedrosis virus), BacH-ARFwt and/or BacH-TopoI (Karayan *et al.*, 2001) and proteins were prepared according to a previously reported protocol (Ayrault *et al.*, 2003). DNA relaxation assays were performed as previously described (Ayrault *et al.*, 2003).

In reconstitution experiments, Topo I was incubated with DNA relaxation reaction mixture for 30 min at 37°C and p14<sup>ARF</sup> was added at different times indicated on the figure. In preincubation experiments, p14<sup>ARF</sup> was incubated to the DNA relaxation reaction mixture at several times before addition of TopoI for 10 min at 37°C. In both cases, protein extracts from cells infected with ACNPVwt were added in the reaction as negative control.

### Cellular protein fractionation

Cells (1.5 × 10<sup>7</sup>) from H-358 or 293 cell lines, were washed in PBS, before extraction in cytoskeleton buffer (CSK): 10 mM Pipes pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl<sub>2</sub>, 1 mM EGTA, 1 mM DTT, and 0.5% (vol/vol) Triton X-100, supplemented with 1 mM PMSF and protease inhibitor cocktail (PIC, Pharmingen). After 3 min at 4°C, the cytoskeletal frameworks were separated from soluble proteins by centrifugation at 5000 g for 3 min. The resulting pellet was washed twice with CSK buffer before chromatin was solubilized in CSK buffer plus proteinase inhibitors, and digested with 1 mg/ml of RNase-free DNase I for 15 min at 37°C. Ammonium sulfate was added to a final concentration of 0.25 M and, after 5 min at 4°C, samples were precipitated. The pellets were further extracted with 2 M NaCl in CSK buffer for 5 min at 4°C, and then centrifuged. The remaining pellets were solubilized in urea buffer and were referred to as the nuclear matrix fractions. Each fraction was dialysed overnight at 4°C against TE buffer (Tris-HCl pH 7.4 10 mM, EDTA 1 mM). The total protein concentrations were determined by the BCA kit (Bicinchoninic Acid, SIGMA). Proteins were separated on denaturing polyacrylamide gels, transferred to immobilon-P and detected by immunoblotting with a goat polyclonal antibody to p14<sup>ARF</sup> (C-18, Santa Cruz), a mouse monoclonal antibody to Rho (gift of Dr N Bourmeyster), or a rabbit polyclonal antibody to Topo I (Topogen). Visualization was

performed by enhanced chemiluminescence according to the manufacturer's instructions (ECL detection kit, Amersham).

#### Coimmunoprecipitation

Fractionation of cellular protein extracts from 10<sup>7</sup> cells (H-358) resulted in four fractions designated soluble proteins, chromatin-associated proteins and proteins linked to the insoluble nuclear matrix. Each fraction was supplemented with 0.1% SDS then precleared with 20  $\mu$ l of protein-A agarose beads (Santa Cruz) and incubated overnight with rabbit polyclonal 73SA anti-ARF serum (Della Valle *et al.*, 1997). Complexes were recovered with 20  $\mu$ l of protein-A agarose and washed with cold wash buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.1% Tween-20 and 1 mM PMSF). Precipitates were separated by SDS-PAGE, followed by TopoI immunodetection.

#### Chromatin fragmentation

Cells (10<sup>7</sup>) from H-358 or 293 lines, were washed twice with PBS, then incubated with 200  $\mu$ l of HNB buffer (15 mM Tris-HCl pH 7.5, 60 mM KCl, 0.25 mM EDTA, 0.125 mM EGTA, 0.5 M sucrose, 0.5 mM spermidine, 0.15 mM spermine, 1 mM DTT, 0.5 mM PMSF, 1/50 PIC-before addition of 100  $\mu$ l of HNB + 1% NP-40 for 5 min at 4°C. Nuclei were pelleted by centrifugation for 3 min at 3200 g.

Nuclei were then incubated in 200  $\mu$ l of micrococcal digestion buffer (20 mM Tris-HCl pH 7.5, 70 mM NaCl, 20 mM KCl, 5 mM MgCl<sub>2</sub>, 3 mM CaCl<sub>2</sub>) and 3 U of micrococcal nuclease for 5 min at room temperature. The reaction was stopped by adding 5 mM EDTA + 5 mM EGTA.

Digestion products were analysed on a 5–30% sucrose gradient by ultracentrifugation for 18 h 20 000 r.p.m. and at 4°C (Beckman, rotor SW-50). Five fractions were recovered, and divided into two subfractions in order to analyse both DNA and proteins. DNA was precipitated with isopropanol 2 h at –20°C, washed with 70% ethanol and dissolved in TE buffer before analysing in 1% agarose gel.

Proteins dialysed against TE buffer (4  $\times$  30 min) sonicated (Branson Sonifier), and acetone precipitated. After centrifugation (20 000 g, 30 min), protein pellets were dissolved in Laemmli buffer and separated by SDS-PAGE. Immunodetection was performed as previously described.

#### Formaldehyde-crosslinking and chromatin immunoprecipitation

293 cells were treated with formaldehyde to a final concentration of 1% for 10 min at 37°C. Crosslinking was stopped by addition of glycine to a final concentration of 0.125 M. Crosslinked cells were harvested, washed in PBS supplemented with 1 mM PMSF. Subsequent procedures were performed on

ice, with buffers supplemented with 1 mM PMSF and PIC. Cells were lysed in lysis buffer (150 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS) for 10 min. Chromatin was sonicated with 10 20-s pulses before centrifugation. The resulting supernatant was diluted 10-fold with dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.1, 167 mM NaCl). Diluted extracts were precleared with protein A agarose beads (Santa Cruz), incubated with 73SA antiserum against p14<sup>ARF</sup> (1/50) (Della Valle *et al.*, 1997) or no antibody (as control), and immunoprecipitated with protein A agarose. Following extensive washes, bound DNA fragments were eluted by overnight incubation at 67°C followed by proteinase K treatment. PCR amplifications were performed with Red Taq genomic (Sigma) in GeneAmpR PCR system 9700 Perkin-Elmer.

Briefly, semiquantitative PCR experiments were first tested onto DNA prepared for ChIP experiments as previously described, but without immunoprecipitation (input). The number of cycles that corresponded to the linear portion of the amplification curve was determined. Under these conditions, the quantity of DNA amplified was two-fold higher when the quantity of matrix was doubled. Quantification was performed by scanning analysis (Scion Image Beta 4.02 software) of 2.5% agarose gel after migration of the amplification products. The same conditions were utilized in parallel with the DNA preparation previously immunoprecipitated with the anti-p14<sup>ARF</sup> 73SA serum (or without antibodies for the control).

18S forward:	5'-ttaaacgaggatccattgga-3'
18S reverse:	5'-ttactcgggaattccctcgt-3'
5.8S forward:	5'-aagcgacgctcagacagcggt-3'
5.8S reverse:	5'-cgactcttagcgggtgatcac-3'
5S forward:	5'-aaagcctacagcaccgggtat-3'
5S reverse:	5'-tctacgccataaccaccctgaacg-3'
GAPDH forward:	5'-gtattccccagggtttatcatg-3'
GAPDH reverse:	5'-ttctgtcttccactcactcc-3'
rRNA promoter forward:	5'-gtttttggggacaggtgt-3'
rRNA promoter reverse:	5'-ccagaggacagcgtgtcagca-3'

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