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The interferon-inducible nucleolar p204 protein binds the ribosomal RNA-specific UBF1 transcription factor and inhibits ribosomal RNA transcription

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p204, a member of the interferon-inducible p200 family of murine proteins, is primarily nucleolar. We generated cell lines in which p204 was inducible by muristerone. This induction resulted in retardation of cell proliferation and inhibition of rRNA transcription *in vivo*. Interferon treatment, resulting in p204 induction and retardation of proliferation, also caused inhibition of rRNA transcription *in vivo*. p204 also inhibited rRNA transcription *in vitro*. This inhibition was overcome by addition of UBF1, the rRNA-specific transcription factor. A direct interaction between p204 and UBF1 was revealed *in vitro* in pull-down assays, and *in vivo* by co-immunoprecipitation from cell extracts. UBF1 bound strongly to at least two regions of p204: the N-terminal segment linked to the conserved 200 amino acid a segment, and the conserved 200 amino acid b segment. Cleavage of the a or b segments into two segments (encoded by single exons) resulted in a strong decrease or loss of binding. The inhibition of rRNA transcription by p204 may be due to the inhibition by p204 of the specific DNA binding of UBF1. This was revealed in electrophoretic mobility shift, magnetic bead and footprinting assays. Thus, p204 serves as a mediator of the inhibition of rRNA transcription by interferon.

Keywords: interferon/p204/rRNA/transcription/UBF

Introduction

The interferons (IFNs) are a family of secreted proteins occurring in vertebrates (Lengyel, 1982; De Maeyer and De Maeyer-Guignard, 1988; Sen and Ransohoff, 1998; Stark *et al*., 1998). They have antimicrobial, cell growth regulatory and immunomodulatory activities. They bind to cell surface receptors and activate the expression of many genes. The proteins encoded by these genes perform the various activities of the IFNs.

Among the proteins induced by IFNs are the members of the p200 family in mice (p202, p203, p204 and D3) (Choubey *et al*., 1989; Choubey and Lengyel, 1992, 1993; Tannenbaum *et al*., 1993; Lengyel *et al*., 1995; Gribaudo *et al*., 1997; Landolfo *et al*., 1998) and in humans (MNDA, IFI16 and AIM2) (Briggs *et al*., 1992, 1994; Trapani *et al*., 1994; Dawson *et al*., 1995, 1996; DeYoung *et al*., 1997). These proteins share at least one 200 amino acid long, partially conserved segment of either the a or b type. Some of the members (e.g. p202, p204 and IFI16) have two such segments, one a and one b type.

The best-characterized protein among these is p202. If even slightly overexpressed in cultured cells, it is growth inhibitory (Choubey and Lengyel, 1993; Min *et al*., 1996). p202 is a primarily nuclear, chromatin-associated protein that inhibits the activity of numerous transcription factors including c-Fos, c-Jun, E2F1, E2F4, MyoD, myogenin, p53, NF-κB p50 and p65 (Choubey *et al*., 1996; Datta *et al*., 1996, 1998; Min *et al*., 1996; Choubey and Gutterman, 1997). In most (but not all) cases, p202 directly binds to these proteins and inhibits their sequence-specific binding to DNA. Inhibition of the expression of p202 by an antisense RNA increases apoptosis in serum-starved AKR-2B cells (Koul *et al*., 1998). p202 is also induced in the course of skeletal muscle differentiation (Datta *et al*., 1998). D3 is inducible in macrophages by lipopolysaccharide (or IFN) (Tannenbaum *et al*., 1993). Its expression is restricted to myeloid cells at or beyond the promyelocyte stage (Weiler *et al*., 1999). Myeloid nuclear differentiation antigen (MNDA), which is present in late myeloid precursors and mature myeloid cells, binds the nuclear proteins nucleolin, nucleophosmin and YY1, increases the affinity of YY1, a transcription factor, for its target DNA and forms a ternary complex with YY1 and DNA (Xie *et al*., 1998). IFI16 is present in myeloid precursors ($CD34^+$) and throughout monocyte development, but its expression is down-regulated in erythroid and polymorphonuclear precursor cells. If fused to the Gal-4 DNA-binding domain, IFI16 functions as a transcriptional repressor (Dawson *et al*., 1998; Johnstone *et al*., 1998).

p204, another member of the murine p200 family, is primarily nucleolar (Choubey and Lengyel, 1992; Lengyel *et al*., 1995). Its overexpression inhibits cell proliferation (Choubey and Lengyel, 1992; Lengyel *et al*., 1995; Lembo *et al*., 1998). It was suggested that p204 may be involved in the differentiation of the myelomonocytic lineage (Gariglio *et al*., 1998). p204's nucleolar location and its antiproliferative activity, together with the fact that the p204-related protein p202 binds to and inhibits the transcriptional activity of numerous transcription factors, prompted the studies reported herein. These were initiated to establish whether p204 inhibits the nucleolar transcription of rRNA.

This process is catalyzed by RNA polymerase I, a multiprotein complex, together with several further proteins (Bell *et al*., 1990; Schnapp and Grummt, 1991; Comai *et al*., 1992; Wolffe, 1994; Hannan and Rothblum, 1995; Mason *et al*., 1997; Seither *et al*., 1998). These include the UBF (upstream binding protein) transcription factor (Bell *et al*., 1988; Hisatake *et al*., 1991; Kuhn and Grummt, 1992). UBF can dimerize, it has an acidic C-terminus and has five domains homologous to the nuclear

HMG1 protein. HMG proteins (e.g. UBF) can bind to crossed DNA helices (e.g. presented by nucleosome-exiting DNA in chromatin) or cruciform DNA, and they induce bends in DNA (Jantzen *et al*., 1990; Maeda *et al*., 1992; Kuhn *et al*., 1994; Putnam *et al*., 1994). The extended footprint of the UBF protein on rDNA is consistent with DNA wrapping around a UBF dimer (Kuhn *et al*., 1994; Putnam *et al*., 1994). UBF can bind to DNA of diverse sequence. Thus it binds to the rDNA core promoter, an upstream control domain located –100 to –160 nucleotides upstream of the rRNA transcription initiation site, and the 140 bp repeats in the mouse rDNA spacer (Kuhn *et al*., 1990, 1992, 1994; Paalman *et al*., 1995). UBF1 interacts with the murine protein complex TIF-1B (designated as SL1 in humans) that consists of TATA box-binding protein (TBP) and three TBP-associated factors (TAFs) (Jantzen *et al*., 1992; Comai *et al*., 1994; Zomerdijk *et al*., 1994; Beckmann *et al*., 1995; Hempel *et al*., 1996; Kihm *et al*., 1998). UBF promotes the binding of TIF-1B to the rDNA core promoter, and the resulting complex promotes the binding of polymerase I (Bell *et al*., 1988; Beckmann *et al*., 1995). UBF1 activity is affected by serum-dependent serine residue phosphorylation (O'Mahony *et al*., 1992; Voit *et al*., 1992, 1995; Kihm *et al*., 1998). TIF-1A, a growth-regulated factor, is also involved in rRNA chain initiation, whereas another factor, TIF-1C, is involved in both initiation and elongation (Schnapp *et al*., 1993, 1994). Three proteins (PAF53, 51 and 49) are also associated with murine RNA polymerase I (Hanada *et al*., 1996). Of these, PAF53 interacts with UBF *in vitro*. The relationship of the three PAFs to TIF-1A and 1C will require further clarification.

The negative growth regulator and tumor suppressor protein pRb was found to accumulate in the nucleoli of differentiating monocytes, and to inhibit rRNA transcription by binding to UBF1 and interfering with its binding to rDNA (Cavanaugh *et al*., 1995; Herwig and Strauss, 1997; Voit *et al*., 1997). Two of the 'HMG boxes' of UBF1 bind to pRb, whose C-terminal segment is involved in the interaction (Voit *et al*., 1997).

Here we report data indicating that the nucleolar p204 protein inhibits rRNA transcription *in vivo* and *in vitro*, by binding to the UBF transcription factor and inhibiting its specific binding to the rDNA regulatory regions. p204 is the first IFN-inducible protein found to affect rRNA synthesis. The inhibition of this process by p204 may contribute to the inhibition of cell proliferation by the IFNs.

Results

Generation of cell lines in which the expression of p204 can be selectively induced

As noted, earlier observations revealed that overexpression of p204 in cultured cells inhibits cell proliferation (Choubey and Lengyel, 1992; Lengyel *et al*., 1995; Lembo *et al*., 1998). To explore this phenomenon further, we used a kit (Invitrogen) to generate cell lines in which p204 was inducible by the ecdysone analog, muristerone (Mur). For this purpose, we placed p204 cDNA downstream of an ecdysone response element in an expression vector and co-transfected this construct with a plasmid encoding subunits of the heterodimeric nuclear Mur receptor into AKR-2B cloned murine embryo cells. Two stable

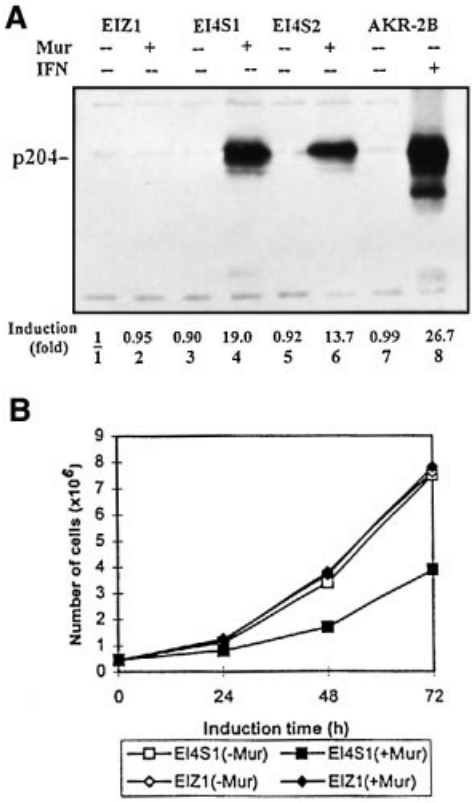

Fig. 1. Induced p204 inhibits cell proliferation. (**A**) Treatment of the EI4S1 and EI4S2 cell lines with muristerone (Mur) induces p204. The two cell lines were derived from AKR-2B cells by the transfection of constructs making the expression of p204 inducible by Mur. The EIZ1 control line in which Mur induces β-galactosidase, but not p204, was also derived from AKR-2B cells. The cell cultures specified, if so indicated (+), were treated with 2.5 μM Mur for 48 h (lanes 2, 4 and 6), or with 1000 U/ml IFN-α for 48 h (lane 8); the other cell cultures (–) served as controls (lanes 1, 3, 5 and 7). Total protein was extracted and 10 μg protein aliquots from each sample were analyzed by Western blotting using anti-p204 antiserum. The p204 band is indicated. (**B**) Induced p204 retards cell proliferation. EI4S1 cells and EIZ1 control cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and 15% FBS. When reaching 30% confluency, some of the cultures, as indicated (+Mur), were supplemented with 2.5 μM Mur (0 time). After further incubation, the cells were counted in a Coulter counter at the times indicated. For further details, see Materials and methods.

cell lines were selected, EI4S1 and EI4S2, in which treatment with Mur resulted in a 21- and 15-fold increase in p204 levels, respectively (Figure 1A). Mur did not induce p204 in the control line, EIZ1 (in which β-galactosidase expression was put under Mur control). The level of p204 induced by Mur was lower than that induced by IFN treatment (27-fold) in AKR-2B cells. This reveals that the Mur-induced level of p204 in our experiments was not unphysiologically high.

Inhibition of cell proliferation by the Mur-induced increase in p204 level

The Mur-induced increase in the p204 level inhibited the proliferation of EI4S1 cells (Figure 1B). The inhibition was slight (27%) 24 h after the addition of Mur to the medium, but increased to 50% by 48 h, and remained at this level at 72 h. Cell proliferation was not affected by Mur in cells (EIZ1) in which β-galactosidase (but not p204) was inducible by Mur. The time course of the Mur-

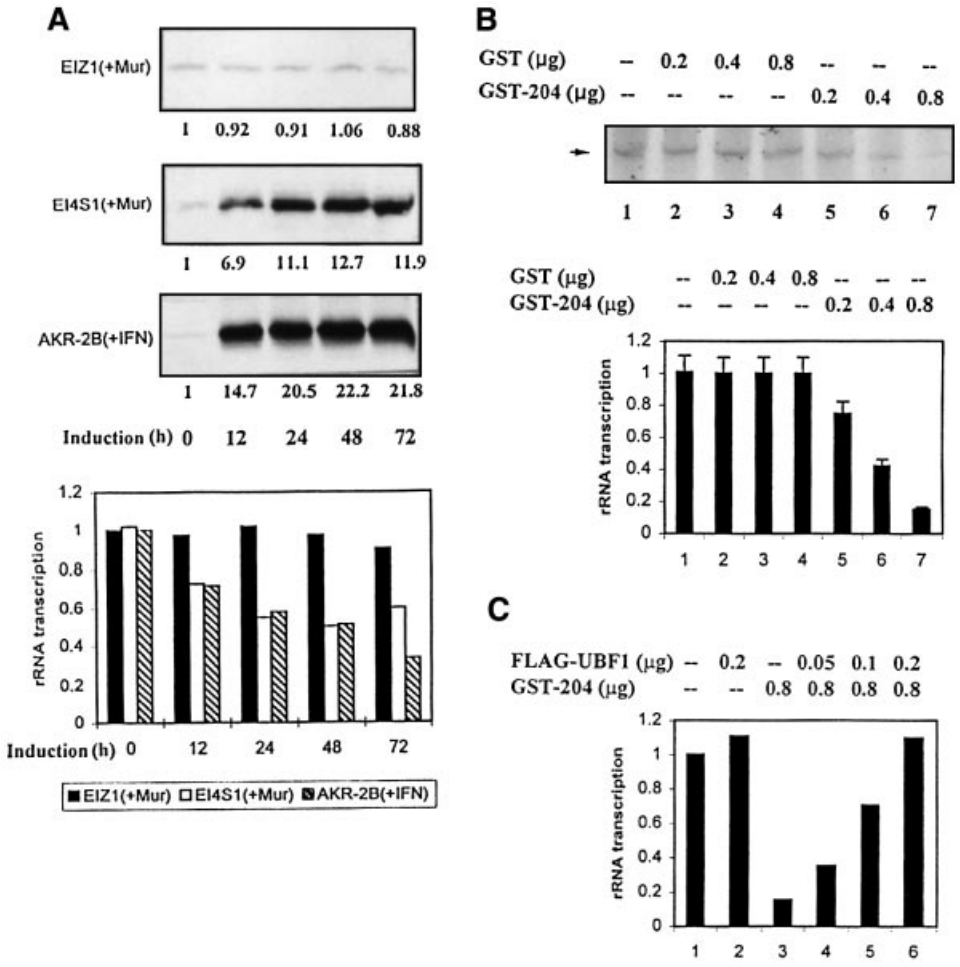

Fig. 2. p204 inhibits rRNA transcription *in vivo* and *in vitro*. Added UBF1 overcomes this inhibition *in vitro*. (**A**) Induced p204 inhibits rRNA transcription *in vivo*. Upper panel: induction of p204 in EI4S1 cells but not in EIZ1 control cells by 2.5 μM Mur and induction of p204 in AKR-2B cells by 1000 U/ml IFN. The extent of p204 induction was determined at the times indicated by immunoblotting with anti-p204 polyclonal antiserum and densitometry. The fold induction is specified. Lower panel: inhibition of rRNA transcription in the EI4S1 cell line treated with Mur and in the AKR-2B cell line treated with IFN. Nuclear run-on assay. The EIZ1, EI4S1 and AKR-2B cell lines were cultured in growth medium (DMEM, 10% FBS). At 0 time, the EIZ1 and EI4S1 cultures were supplemented with 2.5 μM Mur, and the AKR-2B culture with 1000 U/ml IFN. At the times indicated, nuclei were isolated from the three cultures and nuclear run-on analysis was performed in reaction mixtures (including one ^{32}P-labeled and three unlabeled rNTPs) in order to establish the amount of rRNA transcribed. ^{32}P-labeled rRNA transcripts were detected by hybridization to immobilized rDNA and autoradiography, and assayed by laser densitometry. (**B**) p204 dosage-dependent inhibition of rRNA transcription *in vitro*. A segment from the 5′-terminal region of the murine rDNA gene was transcribed in a nuclear extract from L1210 mouse lymphoblastic leukemia cells that was supplemented with one ^{32}P-labeled and three unlabeled rNTPs, without or after addition to the reaction mixtures (25 μl) of the indicated amounts of purified GST–p204 fusion protein or GST protein (serving as a control). The resulting labeled 514 nucleotide transcript was extracted and analyzed by gel electrophoresis, autoradiography and densitometry. Upper panel: autoradiogram from one of three experiments. Lower panel: diagram of the average from three experiments with the standard deviation indicated. (**C**) Added UBF1 (actually FLAG-UBF1) overcomes the inhibition of rRNA transcription by p204 *in vitro*. The assays were performed and analyzed as in (B), except that the reaction mixtures were supplemented with the indicated amounts of FLAG-UBF1 and/or GST–p204. For further details, see Materials and methods.

induced change in p204 levels (Figure 2A, upper panels) reveals a rapid, pronounced (7-fold) increase by 12 h, and further increases by 24 (11-fold) and 48 h (13-fold).

Inhibition of rRNA transcription by p204 in vivo and in vitro

The facts that (i) p204 is located primarily in the nucleolus, the site of rRNA transcription (Choubey and Lengyel, 1992), and (ii) p204 inhibited cell proliferation (Choubey and Lengyel, 1992; Lengyel *et al*., 1995; Lembo *et al*., 1998) prompted us to test whether p204 may also inhibit rRNA transcription.

The data in Figure 2 reveal that this was indeed the case. The rapid increase in p204 level upon exposure of the EI4S1 (but not the EIZ1 controls) culture to Mur is shown in Figure 2A (upper panels). This induction of p204 in the EI4S1 line by Mur resulted in the inhibition of rRNA transcription as tested in a nuclear run-on assay. The inhibition was 32% at 12 h, and increased to the maximal level, 47%, by 48 h (Figure 2A, lower panel). Since p204 is also inducible by IFN (Figure 2A, upper panel), and the induced p204 accumulates primarily in the nucleolus, it was expected that treatment with IFN should also inhibit rRNA transcription. This expectation was verified in a nuclear run-on assay with nuclei from IFN-treated and control AKR-2B cultures (Figure 2A, lower panel). After 72 h of IFN treatment, the extent of inhibition was higher than in the case of the Mur treatment of the EI4S1 cells. This might be as a consequence of the fact that in this set of experiments IFN induced a higher level

of p204 than Mur, and IFN could have altered the expression of many other genes that Mur did not affect (Choubey and Lengyel, 1992; Lengyel *et al*., 1995; Lembo *et al*., 1998; Sen and Ransohoff, 1998).

p204 also inhibited rRNA transcription *in vitro*. The addition of purified glutathione *S*-transferase (GST)–p204 (but not of GST) to a nuclear extract in which a segment from the 5′-terminal region of murine rDNA was transcribed resulted in a dosage-dependent inhibition of rRNA accumulation (Figure 2B). These results suggest that the inhibition of rRNA synthesis *in vivo* by p204 was not only a consequence of the inhibition of cell proliferation by p204.

As noted earlier, rRNA transcription is inhibited by pRb (the negative growth regulator and tumor supressor) which binds UBF1, the rRNA-specific transcription factor (Cavanaugh *et al*., 1995; Herwig and Strauss, 1997; Voit *et al*., 1997). Futhermore, the p204 homolog p202 protein inhibits polymerase II transcription by binding to various sequence-specific transcription factors (Choubey *et al*., 1996; Datta *et al*., 1996, 1998; Min *et al*., 1996; Choubey and Gutterman, 1997). These facts prompted us to explore whether p204 might inhibit rRNA transcription by binding to and sequestering UBF1.

To explore this possibility, we tested whether excess UBF1 can overcome the inhibition of rRNA transcription by p204 *in vitro*. The data in Figure 2C reveal that it could. UBF1 (actually FLAG-UBF1) added to a nuclear extract increased the *in vitro* transcription of rRNA only slightly (~10%). Added p204 (actually GST–p204) inhibited this process by ~90%. Further addition of UBF1 (to the reaction mixture already supplemented with p204) could fully overcome the inhibition in a dosage-dependent manner.

Interaction of p204 with the rDNA-specific UBF1 transcription factor in vitro and in vivo

Finding that excess UBF1 could fully overcome the inhibition of rRNA transcription *in vitro* by p204 prompted us to test whether p204 can bind UBF1. The data in Figure 3 indicate that it can. FLAG-tagged UBF1 (but not the FLAG peptide itself) bound p204 in a pull-down assay, revealing an interaction between the two proteins *in vitro* (Figure 3A). Furthermore, a polyclonal anti-p204 antiserum (but not a pre-immune serum) co-immunoprecipitated UBF1 together with p204 from extracts of IFN-treated AKR-2B cells, or Mur-treated EI4S1 and EI4S2 cells, although not from control AKR-2B cells (in which the level of p204 was expected to be too low) (Figure 3B). A DNA bridge was not required for the interaction of p204 and UBF1: the binding between the two proteins persisted even (i) after incubation of the reaction mixtures with DNase I or (ii) in the presence of ethidium bromide (an agent known to inhibit protein DNA association; not shown) (Lai and Herr, 1992).

Identification of p204 segments binding UBF1

A series of truncated and cleaved derivatives of p204 were generated and used to characterize the sites of interaction between p204 and UBF1 (Figure 4A and C). As revealed in Figure 4B and indicated in Figure 4A, the N-terminal 30 amino acids were not required for the binding. The removal of the b segment diminished the

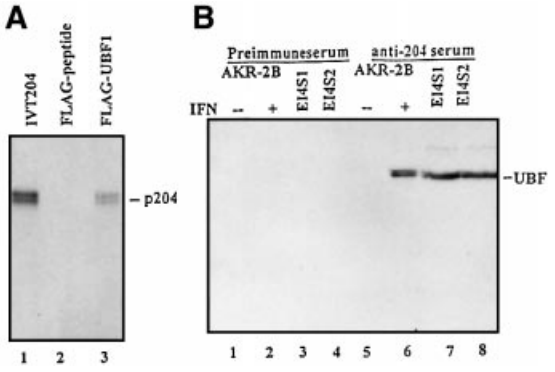

Fig. 3. p204 interacts with the rDNA-specific UBF1 transcription factor *in vitro* and *in vivo*. (**A**) FLAG-tagged UBF1 pull-down assay for testing the binding of p204 *in vitro*. [^{35}S]methionine-labeled p204 translated in a reticulocyte lysate (lane 1), binding to FLAG peptide bound to the M2 beads (lane 2) or to FLAG-UBF1 bound to M2 beads (lane 3). The bound protein was analyzed by gel electrophoresis and fluorography. The position of the p204 band is indicated. (**B**) Binding of p204 to UBF1 *in vivo* as assayed by co-immunoprecipitation. Extracts from control (lanes 1 and 5) or IFN-treated AKR-2B cells (lanes 2 and 6) or from control (lanes 3 and 4) or Mur-treated EI4S1 or EI4S2 cells overexpressing p204 (lanes 7 and 8) were co-immunoprecipitated with pre-immune serum (lanes 1–4) or anti-p204 antiserum (lanes 5–8). The immunoprecipitates were examined by immunoblotting with anti-UBF1 antiserum. The UBF band is indicated. For further details, see Materials and methods.

binding. The cleavage of the residual p204 segment into the N-terminal and a segments did not decrease the binding further, whereas an additional cleavage of the 5′ a segment into exonic segments did. Neither the N-terminal segment alone nor the a segment alone bound UBF1, whereas the b segment alone did bind, unless cleaved into exonic segments.

These results indicate the binding of UBF1 to at least two regions of p204: (i) the N-terminal plus a segment (even when these were cleaved apart) and (ii) the b segment.

Inhibition of the binding of UBF1 to cruciform DNA by p204

UBF1 belongs to a family of proteins whose DNA-binding domains are homologous to the non-specific DNA-binding domains of HMG proteins 1 and 2 (Jantzen *et al.*, 1990; Kuhn *et al*., 1994). The natural target DNA segment of mouse UBF1 is the mouse rDNA promoter. However, it was reported that UBF1 (like HMG1) can bind to synthetic four-way junction DNA (cruciform DNA) of non-target-related sequence (Bianchi, 1988), and binds to such DNA with higher affinity than to the linear duplex DNA of its authentic target (Kuhn *et al*., 1994). Using electrophoretic mobility shift asssays (EMSAs), we verified that our FLAG-UBF1 preparation bound cruciform DNA (Figure 5A, lane 3). This binding was inhibited by excess, unlabeled cruciform DNA (lane 5), but not by non-specific DNA (lane 4), and an antiserum to UBF1 supershifted the UBF1–cruciform DNA complex (lane 6). As reported earlier, GST–pRb inhibited this binding (Voit *et al*., 1997) (Figure 5B, compare lane 2 with lanes 7–9) as did GST–p204 in a dosage-dependent manner (compare lane 2 with lanes 3–5).

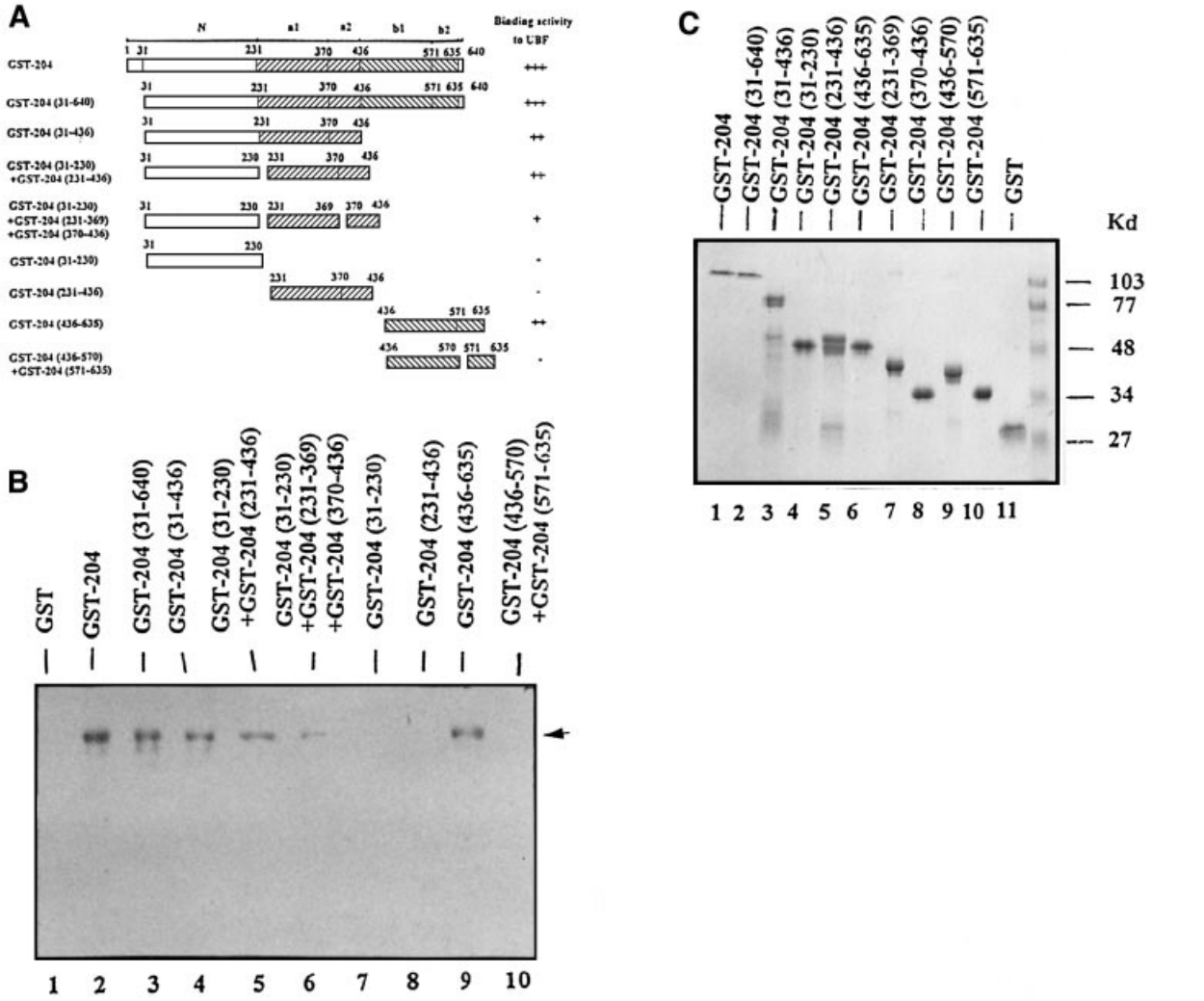

Fig. 4. Identification of p204 segments binding to UBF1. (**A**) Schematic diagram of GST–p204 constructs used to map the binding of p204 to UBF1. Numbers refer to the amino acid residues in p204. N, N-terminal segment. a_1, a_2, b_1, b_2 segments encoded by single exons. The strength of the binding activities as revealed in (B) is indicated. (**B**) Binding of UBF1 by p204, its segments as well as their combinations. Glutathione–Sepharose beads carrying GST, GST–p204 or its segments, as indicated, were incubated with FLAG-UBF1 and the bound FLAG-UBF1 was released and detected by immunoblotting with an anti-FLAG probe. The FLAG-UBF1 is indicated by an arrow. (**C**) Expression of GST linked to p204 or segments of p204. GST linked to p204 or its segments, as indicated, was expressed in *E.coli*, purified, and 0.5 μg of protein from each was examined by SDS–PAGE and Coomassie blue staining. The positions of size markers in kDa are indicated. For further details, see Materials and methods.

Inhibition of the binding of UBF1 to the murine rDNA promoter by p204

Magnetic bead assay. A ribosomal promoter segment was biotinylated and loaded onto streptavidin-coupled magnetic beads. FLAG-UBF1 was bound to the loaded (but not to the unloaded) beads (Figure 6A, lanes 1 and 2); the binding was inhibited by an excess of non-biotinylated DNA segment (lane 3), but not by an excess of non-specific DNA (lane 4). GST–p204 (but not GST) inhibited the sequence-specific binding of UBF1 to DNA in a dosage-dependent manner (compare lane 2 with lanes 5–8).

DNase I footprinting assay. The promoter segment was labeled with ^{32}P at its 5′ terminus and, as indicated, was incubated with various concentrations of FLAG-UBF1 and/or GST–p204 prior to digestion with DNase I and electrophoresis in a sequencing gel (Figure 6B, left panel). FLAG-UBF1 protected segments of the DNA from cleavage by DNase I (compare lane 1 with lanes 2–4). GST–p204 did not protect in itself (lane 5); however, it overcame the protection by UBF1 in a dosage-dependent manner (lanes 6–8).

Thus, three types of test clearly indicated that p204 can inhibit the structure (cruciform DNA)- or sequence (rDNA promoter)-specific binding of UBF1 to DNA.

We examined whether p204 can substitute for the structurally similar p202 in a footprinting assay (Figure 6B, right panel). An AP2 transcription factor preparation, as expected, protected its recognition sequence in a segment of the ^{32}P-labeled SV40 early promoter DNA (Mitchell *et al*., 1987) from DNase I cleavage (compare lanes 1 and 2). p202 [the p204 homolog known to inhibit the activity of the AP2 transcription factor (Min *et al*., 1996)] did overcome this protection (lane 6), whereas GST–p204 (not known to interact with the AP2 protein) did not (lane 5).

Discussion

The Mur-inducible mammalian expression system (Invitrogen) turned out to have characteristics appropriate for

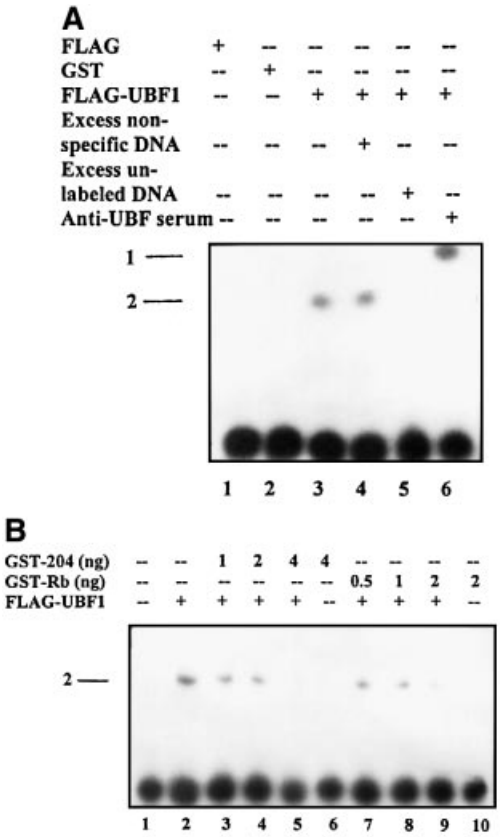

Fig. 5. p204 inhibits the binding of UBF1 to cruciform DNA. EMSA. (**A**) Specific binding of UBF1 to cruciform DNA. Proteins (FLAG, GST or FLAG-UBF1) were incubated with labeled cruciform DNA without or with added 50-fold excess of non-specific DNA (sonicated salmon sperm DNA from Promega), or unlabeled cruciform DNA or anti-UBF antiserum as indicated. The positions of the UBF1–cruciform DNA complex (2) and of the UBF1–cruciform DNA complex supershifted by an anti-UBF antiserum (1) are indicated. (**B**) GST–p204 and GST–pRb fusion proteins inhibit the specific binding of UBF1 to cruciform DNA. UBF1 (10 ng) was incubated with labeled cruciform DNA with increasing amounts of GST–p204 or GST–pRb as indicated. The position of UBF1–cruciform DNA (2) is indicated. For further details, see Materials and methods.

p204 induction. It did not affect the level of constitutive p204 expression, and maximal induction was lower than that by IFN, ensuring that the level of p204 induced was within the physiological range. Experiments with cell lines in which p204 was inducible by Mur revealed that the increase in p204 level resulted in the inhibition of cell proliferation, as well as the inhibition of rRNA transcription *in vivo*. The fact that p204 also inhibited rRNA transcription *in vitro* (i.e. in nuclear extracts) indicated that the inhibition of rRNA transcription was not a consequence of the retardation of cell proliferation. Added UBF1 could overcome the inhibition of rRNA transcription by p204 *in vitro*, prompting tests on the interaction between p204 and UBF1. FLAG-UBF1 bound p204 *in vitro*, and the fact that an antiserum to p204 co-immunoprecipitated UBF1 together with p204 from a cell extract indicated that the two proteins also interacted *in vivo*. Three types of experiment revealed that p204 can inhibit the specific binding of UBF1 to DNA: EMSA, magnetic bead assays and DNase I footprinting assays. This third type of assay also revealed the specificity of the effect: p204, which blocked the binding of UBF1 to its target rDNA, did not impair the binding of AP2 to its target SV40 DNA (Mitchell *et al.*, 1987). The p204 homolog, p202 (known to inhibit transcription by AP2) (Min *et al.*, 1996) did, however, inhibit the binding of AP2 to its target DNA.

In line with a mechanism in which p204 binds UBF and thereby impairs its binding to DNA, pre-incubation of p204 with UBF1 (in a nuclear extract, prior to the addition of rDNA) resulted in a 50% stronger inhibition of rRNA transcription than pre-incubation of rDNA with UBF1 (in a nuclear extract, prior to the addition of p204) (not shown).

p202 also bound UBF1 (although 11-fold more weakly than p204), and also inhibited rRNA transcription *in vitro* (although a 3.3-fold higher concentration of p202 than of p204 was needed to inhibit the transcription by 50%) (not shown). These findings make it conceivable that p202 might serve as a poor substitute for p204 in inhibiting rRNA transcription *in vivo*. The fact that p202 is a primarily nucleoplasmic protein that does not accumulate specifically in the nucleoli, however, diminishes the likelihood of such a function for p202.

The fact that p202 bound pRb (Choubey and Lengyel, 1995) prompted us to examine whether p204 did too. Both GST pull-down assays and the yeast two-hybrid assay revealed that this was the case (not shown). The mode of inhibition of rRNA transcription by p204 is apparently identical to that by pRb. pRb blocks rRNA synthesis by binding to UBF1 and inhibiting its sequence-specific binding to DNA (Voit *et al.*, 1997). Our experiments examining the extent of the inhibition of rRNA synthesis *in vitro* by pRb together with p204 indicated additivity rather than synergy between the inhibitory activities of the two proteins (not shown).

The results from this study reveal a similarity between the modes of action of p204 and p202, which are both members of the p200 protein family. The two proteins can inhibit the activities of distinct sequence-specific transcription factors by binding to these and inhibiting their sequence-specific binding to DNA.

p204 has been the first IFN-inducible protein found to be localized primarily in the nucleolus (Choubey and Lengyel, 1992), and to inhibit rRNA transcription (this study). It remains to be established whether (i) p204 inhibits rRNA transcription only by binding UBF and inhibiting its binding to DNA and (ii) this inhibition of rRNA transcription can fully account for the retardation of cell proliferation and the impairment of normal embryonic development by p204 (Lembo *et al.*, 1998), or if other, as yet undiscovered, actions of p204 contribute to these effects. It also remains to be explored whether the inhibition of rRNA transcription by p204 contributes to the antimicrobial action of the IFNs directly or by retarding cell proliferation. These effects of p204 might also have a role in the modulation of differentiation by the IFNs. The likelihood of this is enhanced by the reported involvement of several members of the p200 protein family in differentiation (Datta *et al.*, 1998; Dawson *et al.*, 1998; Xie *et al.*, 1998; Weiler *et al.*, 1999). Studies concerning these problems are underway.

Materials and methods

Plasmid constructs

p204 cDNA cloned in pBluescript SK was used for plasmid construction (Choubey *et al.*, 1989). To obtain bacterial expression plasmids encoding

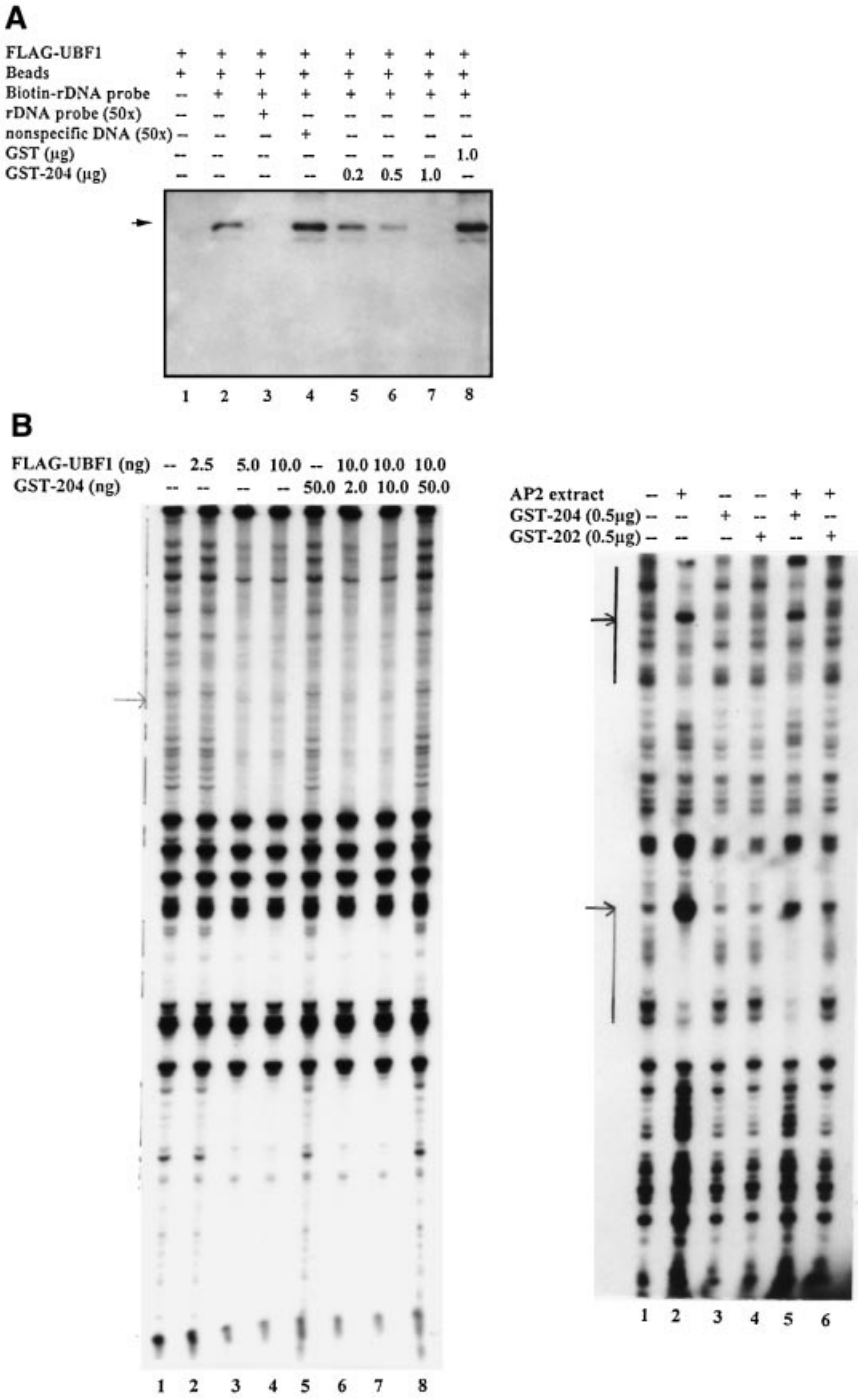

Fig. 6. p204 inhibits the binding of UBF1 to the rDNA promoter. (**A**) Magnetic bead assay. Five pmol of biotin-tagged murine rDNA promoter segment (probe) was conjugated to streptavidin-coupled magnetic beads and incubated with 0.5 μg of FLAG-UBF1 together, if so indicated, with labeled probe, excess not biotin-tagged probe, excess non-specific DNA (sonicated salmon sperm DNA), as well as GST or GST–p204 fusion protein in the amounts indicated, at room temperature, for 20 min. The FLAG-UBF1 bound to the beads was released and analyzed by SDS–gel electrophoresis and immunoblotting with an anti-FLAG antiserum. The FLAG-UBF1 band is indicated by an arrow. (**B**) DNase I footprinting. Left panel: the murine rDNA promoter segment 5′ labeled with ^{32}P at position –169 was incubated on ice with FLAG-UBF1 and/or GST–p204 in the amounts indicated. The reaction mixture was supplemented with DNase I and incubated at room temperature. The isolated DNA was analyzed by electrophoresis on a sequencing gel. Right panel: to test the specificity of the p204 effect, 5′ ^{32}P-labeled SV40 early promoter DNA segment was incubated with AP2 extract and GST–p204 or GST–p202 as indicated. Further processing was as in the left panel. Protected regions are shown with vertical lines next to the sequence. The arrow indicates hypersensitive sites. For further details, see Materials and methods.

GST–p204 fusion proteins, full-length p204 cDNA and a series of p204 cDNA segments were inserted into the pGEX-3X Pharmacia expression vector. Constructs generated are shown in Figure 5A. One additional construct [GST–p204 (129–177)] includes the seven amino acid repeat segment from the N-terminal region of p204. Details of the constructions are available upon request.

Expression and purification of GST fusion proteins and FLAG-tagged UBF1

For expression of GST fusion proteins, the appropriate GST–p204 plasmids (Figure 5A) were introduced into *Escherichia coli* DH5α (Gibco-BRL). The fusion proteins synthesized were affinity purified on glutathione–Sepharose beads (Pharmacia) as described (Smith, 1993),

and their amounts were determined by the dot metric 1 μl protein assay kit (Chemicon). Recombinant human FLAG-UBF1 was produced in High Five (HI5) insect cells (Invitrogen), infected with recombinant baculovirus (Beckmann *et al*., 1995), generously provided by I.Grummt (Voit *et al*., 1997). FLAG-UBF1 was purified according to Voit *et al*. (1997).

Preparation of an antiserum to p204

The GST–p204 fusion protein GST–p204 (129–177) encoding the p204 seven amino acid repeat was expressed in *E.coli* DH5α, purified on a glutathione–Sepharose column and subjected to preparative scale SDS–PAGE. The major band was excised and used to immunize rabbits at the animal facility of Yale University.

Generation of cell lines in which Mur treatment induces p204

The EI4S1 and EI4S2 cell lines were derived from AKR-2B cloned murine embryo cells by the transfection of plasmids (from the Ecdysone-Inducible Expression Kit from Invitrogen) encoding hormone receptors, selectable markers and inducible p204. Stable cloned lines were selected, including EI4S1 and EI4S2 in which p204 is inducible by Mur, as well as a control line EIZ1 in which β-galactosidase (but not p204) is inducible by Mur.

Nuclear run-on analysis

This was performed essentially according to Ausubel *et al.* (1994). Nuclei were isolated from 5×10^7 cells of the stable lines EIZ1 and EI4S1 after or without treatment with Mur, and from AKR-2B cells after or without treatment with IFN (as specified), and were incubated in a total volume of 200 μl with 60 μCi of [^{32}P]UTP (Amersham) and 500 μM ATP, CTP and GTP in a buffer (10 mM Tris–HCl pH 8.0, 300 mM KCl, 5 mM $MgCl_2$) at 30°C for 30 min. The rRNA-specific transcripts were detected by dot hybridization to immobilized 45S rDNA. pUC19 was also immobilized on the same filter to control for non-specific hybridization. The hybridized radioactivity was detected by autoradiography and assayed by a laser densitometer (Molecular Dynamics). Human recombinant α2/α1 (1–83) IFN that is active on murine cells was used (Weber *et al*., 1987).

In vitro transcription assay

Nuclear extract was prepared from mouse lymphoblastic leukemia cells (L1210) (ATCC No. CCL219) as described by Haglund and Rothblum (1987). The assay was performed essentially according to Cassidy *et al*. (1987), except that the volume of the reaction mixture was 25 μl, 5 μl of nuclear extract was used, GST, GST–p204 and FLAG-UBF1 were used as specified and 2 ng of murine rDNA served as the template. This was prepared from plasmid P-2150 (kindly provided by Dr Sollner-Webb) which consisted of the murine rDNA enhancer–promoter region together with a segment encoding the 5′-terminal region of rRNA (Paalman *et al*., 1995). When cleaved with *Nde*I, it encoded a 514 nucleotide transcript (Osheim *et al*., 1996). The reaction mixture was first incubated at 30°C for 20 min. Thereafter, the transcription was initiated by adding 0.5 mM GTP and 25 μM [α-^{32}P]UTP (20 μCi). After incubation at 30°C for 90 min, the reaction mixture was processed according to Cassidy *et al*. (1987). The labeled rRNA transcript was assayed by scanning using a Kodak Digital Image system.

In vitro binding assay

^{35}S-labeled p204 (IVT-204) was expressed in a rabbit reticulocyte transcription–translation system (Promega). To examine the binding of p204 to immobilized FLAG-tagged UBF1 (FLAG-UBF1), 10 μl of IVT-204 was incubated at 4°C for 4 h with 20 μl of packed M_2 beads (from Kodak) loaded with 0.5 μg of FLAG peptide (control), or 0.5 μg of FLAG-UBF1 in 200 μl of buffer AM (Voit *et al*., 1997) supplemented with 100 mM KCl and 0.5 mg/ml bovine serum albumin (BSA). The reaction mixture was processed according to Voit *et al*. (1997). The bound [^{35}S]p204 was analyzed by SDS–PAGE and visualized by autoradiography. To identify p204 segments which bind FLAG-UBF1, glutathione–Sepharose beads loaded with GST (0.5 μg) serving as control, or GST–p204 (0.5 μg) or GST–p204 segments (0.5 μg) were incubated with 0.5 μg of purified FLAG-UBF1 in buffer AM, supplemented with 100 mM KCl and 0.5 mg/ml of BSA at room temperature for 40 min. The bound material was examined by SDS–PAGE. The FLAG-UBF1 was detected by immunoblotting with anti-FLAG antibodies.

Co-immunoprecipitation

AKR-2B cells and cells from the stable lines EI4S1 and EI4S2 (in which p204 is inducible by Mur) were grown in monolayers in 100 mM dishes in DMEM, 10% fetal bovine serum (FBS). When the cultures reached 50% confluency, 1000 U/ml IFN-α or 2.5 μM Mur (Invitrogen) was added, as indicated, and the cultures were incubated for 48 h. Cell extracts were prepared according to Min *et al*. (1996). Aliquots (200 μl) from the cell extracts were incubated at 4°C overnight with 30 μl of packed Affi-gel-10 beads (Bio-Rad) to which an anti-p204 antiserum or pre-immune serum was covalently linked. The beads were then washed with immunoprecipitation (IP) buffer (Min *et al*., 1996) five times and the bound proteins were eluted with IP buffer supplemented with 500 mM NaCl and 1% SDS at room temperature for 2 h. The released proteins were examined by Western blotting with the anti-UBF antiserum (Hannan *et al.*, 1998) (a generous gift from L.Rothblum).

EMSA

Four-way junction DNA was prepared by annealing four appropriate oligonucleotides partially complementary to each other, which assemble into a cruciform molecule. The sequences and the construction of the oligonucleotides were as described by Bianchi (1988). For EMSA, one of the oligonucleotides was labeled with ^{32}P by T4 kinase. The binding assay was performed in a 10 μl volume containing 10 mM HEPES buffer (pH 7.9), 200 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM spermidine, 0.5 mM dithiothreitol (DTT), 8% Ficoll (Sigma), 1 nM labeled DNA probe, 10 ng of recombinant UBF1 and various amounts of GST–p204 or GST–pRb (379–928) as indicated. After incubation at room temperature for 20 min, the samples were subjected to 6.5% PAGE in 0.5% TBE (45 mM Tris–borate, 1 mM EDTA) at 15 V/cm, at room temperature for 1.5 h.

Magnetic bead assay

A 177 nucleotide DNA fragment containing the murine ribosomal promoter region (from nucleotide –169 to +9, prepared from p-2150) was labeled with biotin using the Promega random primed DNA labeling kit (DNA probe). A 5 pmol aliquot of DNA probe was conjugated to 20 μl of streptavidin-coupled magnetic beads (Promega) in BC buffer (20 mM HEPES pH 7.9, 100 mM NaCl, 0.5 mM EDTA, 10% glycerol, 0.01% NP-40) and incubated with 500 ng of FLAG-UBF1 and various amounts of GST–p204, as indicated, in 300 μl of the same buffer at room temperature for 20 min. The bound FLAG-UBF1 was detected by SDS–PAGE and immunoblotting with the anti-FLAG antiserum (Santa Cruz).

DNase I footprinting

Footprinting was performed using the core footprinting system (Promega) with minor modifications. The murine rDNA promoter segment used (Osheim *et al*., 1996) extended from nucleotide –169 to +292 (DNA probe). This was labeled with [γ-^{32}P]ATP and T_4 oligonucleotide kinase at nucleotide –169. The incubation mixture (50 μl) containing 5 ng of 5′-labeled DNA probe was supplemented with purified FLAG-UBF1 or GST–p204 in the amounts indicated, and incubated on ice for 10 min. After digestion with 0.15 U of DNase I for 1 min, the samples were analyzed by electrophoresis on a 6% polyacrylamide, 8% urea sequencing gel and autoradiography.

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