A Transcription-dependent Micrococcal Nuclease-resistant Fragment of the Urokinase-type Plasminogen Activator Promoter Interacts with the Enhancer*

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We show the interaction between the enhancer and the minimal promoter of urokinase-type plasminogen activator gene during active transcription by coupling micrococcal nuclease digestion of cross-linked, sonicated chromatin, and chromatin immunoprecipitation. This approach allowed the precise identification of the interacting genomic fragments, one of which is resistant to micrococcal nuclease cleavage. The interacting fragments form a single transcriptional control unit, as indicated by their common protein content. Furthermore, we show that the enhancer-MP interaction persists during the early stages of transcription and is lost upon α-amanitin treatment, indicating the requirement for active transcription. Our results support a looping model of interaction between the enhancer and the MP of the urokinase-type plasminogen activator gene.

Transcription regulation in eukaryotic cells is a multistep process that involves the assembly of multiprotein complexes on gene regulatory regions (1). These regulatory regions contain two types of sequences: enhancers/silencers, which recruit a complex array of transcription factors and chromatin-modifying activities, and core promoter elements to which the general transcriptional machinery, including RNAP II, is recruited. Understanding the molecular mechanisms involved in transcriptional regulation over long distances is of fundamental importance, because in most cases the activities of remote control elements are essential in turning on or off specific subsets of genes in a temporally and spatially regulated manner (2). The main models to explain distal enhancer function involve enhancer-promoter communication, either through protein-protein interactions resulting in the formation of DNA/chromatin loops (looping model), the free sliding of proteins recruited on the enhancer along the DNA (scanning model), or the establishment of modified chromatin domains between the enhancer and the promoter by facilitator proteins, which generate a progressive chain of higher order complexes along the chromatin fiber (3–9). The looping model, however, is supported by recent results in a number of different experimental systems, showing that the close physical proximity of distant regulatory sequences is required for proper gene expression (10–15). The interaction between enhancer elements and proximal promoter culminates in the transcription activation event. However it is not yet clarified if the activation of transcription stems from the transient interaction of regulatory elements or through a more stable structure working as a single control unit.

The uPA gene codes for a serine protease involved in the degradation of the extracellular matrix and in cell motility (16). The expression of this gene is normally dependent on the presence of inducers, but in cancer cells it is often constitutive. The regulatory region of the uPA gene contains an MP and an enhancer located 2 kb upstream (17). In highly invasive human prostate adenocarcinoma (PC3) cells this gene is constitutively expressed (18) and is present in multiple copies, not all of which are expressed (19), and the activity of the MP is modulated by Sp1 (18, 20). The uPA enhancer contains two AP1 binding sites (21–25) and, together with the MP, is important for high expression levels of the gene (17, 18).

In an attempt to obtain new insights into the molecular mechanism involved in enhancer-mediated gene activation, we asked if specific DNA fragments in the enhancer and MP of the uPA gene were involved in the interaction between the regulatory elements by using a modified chromatin immunoprecipitation (ChIP) approach, including an MNase digestion step prior to immunoprecipitation. This procedure allows the fine mapping of DNA sequences involved in these interactions, while maintaining the information content on their protein composition.

Here we provide experimental evidences for the presence of specific structures in the MP region of the uPA gene that are associated with transcriptional events and for the interaction of one of them with the enhancer. Our results support a model of interaction between the regulatory regions that persists at least through the early steps of elongation.
MNase-resistant Amplicons in the uPA Promoter

**TABLE 1**
List of the primer sets used in this study

| Primer sets | Primers sequences (5' to 3' orientation) | Primers position in uPA sequence | PCR annealing temperature for primer sets (°C) |
|-------------|------------------------------------------|---------------------------------|-----------------------------------------------|
| F8          | TGGCCAGAGGAACTGCTACAGCCCGG              | −1983/−1985                   | 57                                            |
| R11         | GAAATCTAGAGAATGAGAGAGG                 | −1836/−1839                   |                                              |
| F8          | TGGCCAGAGGAACTGCTACAGCCCGG              | −1983/−1985                   | 57                                            |
| R12         | GAACCAAGCAATACAGGAGATGCGG             | −1792/−1816                   |                                              |
| F8          | TGGCCAGAGGAACTGCTACAGCCCGG              | −1983/−1985                   | 57                                            |
| R14         | CTTGAGGCTCGGCTTTGAAATCTTC              | −1731/−1755                   |                                              |
| F5          | GATTTCCGATGAAATTGAAAGAAATGATTC        | −2105/−2081                   | 54                                            |
| R14         | CTTGAGGCTCGGCTTTGAAATCTTC              | −1731/−1755                   |                                              |
| F22         | CAGAATCTGCTGCTGGCTCTTCC               | −645/−623                    | 60                                            |
| R26         | CAGAATCTGCTGCTGGCTCTTCC               | −181/−181                     | 60                                            |
| F26         | GAATTTCCGATGAAATTGAAAGAAATGATTC        | −205/−181                     | 60                                            |
| R31         | GGGACAGCTGAGGACGAGG                  | +114/+97                     |                                              |
| F22         | CAGAATCTGCTGCTGGCTCTTCC               | −645/−623                    | 60                                            |
| R31         | GGGACAGCTGAGGACGAGG                  | +114/+97                     |                                              |
| F21         | CAGAATCTGCTGCTGGCTCTTC                | −700/−678                    | 60                                            |
| R26         | GAAGCTCTGAGGACTTCCTGTTAATTCT         | −181/−205                    | 60                                            |
| F22         | CAGAATCTGCTGCTGGCTCTTCC               | −645/−623                    | 60                                            |
| R27         | CGTAGCCCTACAGAACAGACGCC             | −114/−134                    |                                              |
| F26         | GAATTTCCGATGAAATTGAAAGAAATGATTC        | −205/−181                     | 60                                            |
| R34         | ACCGAGCTCCACTGCTTC                  | +304/+326                    | 60                                            |
| F26         | GAATTTCCGATGAAATTGAAAGAAATGATTC        | −205/−181                     | 60                                            |
| R36         | GAAGCTCTGAGGACTTCCTGTTAATTCT         | +420/+402                    | 60                                            |
| F26         | GAATTTCCGATGAAATTGAAAGAAATGATTC        | −205/−181                     | 60                                            |
| R37         | GAGCGCCAGAGAAGAGG                   | +465/+447                    | 60                                            |
| F25         | GAGCGCCAGAGAAGAGG                   | −313/−292                    | 60                                            |
| R31         | GGGACAGCTGAGGACGAGG                  | +114/+97                     |                                              |
| F7          | GGGAAAGGGGTTGTTGGTTGCTT              | −2024/−206                    | 57                                            |
| R10         | GCCGCTATGTCCCCTGCTTGTCC              | −1874/−1894                   |                                              |
| F3          | GAGCCACCTGCAAGACCAAGAGA             | −2192/−2171                   | 57                                            |
| R6          | CCCGCTAGTGTTCCCTGCTCAAAC            | −2603/−2604                   |                                              |
| F11         | CTGCTGACCTGCAAGAGGTCGTTCT            | −1859/−1836                   | 57                                            |
| R15         | CTCCTGACCTGCAAGAGGTCGTTCT            | −1707/−1730                   |                                              |
| F6          | GCTGCTGACCTGCAAGAGGTCGTTCT            | −2064/−2043                   | 54                                            |
| R10         | GCCGCTATGTCCCCTGCTTGTCC              | −1874/−1894                   |                                              |
| F9          | GCTGCTGACCTGCAAGAGGTCGTTCT            | −1942/−1922                   | 57                                            |
| R12         | GAGCCAACTGAAAGAGGTCGTTCT            | −1816/−1792                   |                                              |
| F7          | GGGAAAGGGGTTGTTGGTTGCTT              | −2024/−206                    | 54                                            |
| R11         | GAAATCTGAGGAGGAGGAGG                  | −1836/−1839                   |                                              |
| F14         | GAAATCTGAGGAGGAGGAGG                  | −1755/−1731                   | 60                                            |
| R26         | GAGCCACCTGCAAGACCAAGAGA             | −181/−205                    |                                              |
| F22         | CAGAATCTGCTGCTGGCTCTTCC               | −114/−134                    | 57                                            |
| F26         | GAATTTCCGATGAAATTGAAAGAAATGATTC        | −205/−181                     | 60                                            |
| R31         | GGGACAGCTGAGGACGAGG                  | +114/+97                     |                                              |
| F32         | GGGACAGCTGAGGACGAGG                  | +194/+213                    | 57                                            |
| R36         | GAAGCTCTGAGGACTTCCTGTTAATTCT         | +420/+402                    |                                              |
| F29         | GCTGCTGACCTGCAAGAGGAGG               | −85/−65                      | 60                                            |
| R31         | GGGACAGCTGAGGACGAGG                  | +114/+97                     |                                              |
| F26         | GAATTTCCGATGAAATTGAAAGAAATGATTC        | −205/−181                     | 60                                            |
| R29         | CTCCTGACCTGCAAGAGGTCGTTCT            | −65/−85                      |                                              |

**EXPERIMENTAL PROCEDURES**

**Cell Cultures, α-Amanitin Treatment, and Chromatin Preparation**—PC3 cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) containing a final concentration of 10% (v/v) fetal bovine serum, 0.2 mg/ml streptomycin, 20 units/ml penicillin, 2 mM glutamine, and 1 mM sodium pyruvate.

α-Amanitin (Sigma) was dissolved in water, and increasing concentrations were added to the culture medium for 24 h, as previously described (26). Inhibition of uPA transcription was estimated by quantitative reverse transcription-PCR of uPA mRNA at different α-amanitin concentrations. At 10 μg/ml uPA transcription was inhibited by >90%; this concentration was used in further experiments as follows. Untreated cells or cells that were treated with α-amanitin (10 μg/ml for 24 h) were cross-linked with 1% formaldehyde for 10 min, and chromatin was prepared essentially as described (27) by using 10 sonication cycles (35 s at 60–70 watts, in an Ultrasonic Processor XL Sonicator (Misonix), followed by a 2-min rest on ice). Cross-linked chromatin-containing fractions were pooled and stored at −80 °C. Cross-linked chromatin was prepared from different batches of PC3 cells (two batches of untreated and two batches of α-amanitin-treated) PC3 cells. All experiments (e.g. MNase digestion and ChIP) were repeated at least twice for each batch.

**RNA Detection**—Total RNA was extracted with an RNeasy mini kit (Qiagen), quantitated by spectrophotometry (Nanodrop), and 5 μg was reverse-transcribed using a SuperScript™ First-Strand kit using random primers (Invitrogen) according to the manufacturer’s instructions.

For quantitative reverse transcription-PCR, 5 ng of reverse-transcribed RNA were amplified, and the amplification products were detected using the TaqMan gene expression assay (Hs00170182_m1) primers and probes specific for uPA, in an ABI PRISM 7900HT Sequence Detection System. 18 S rRNA gene levels were examined and used to normalize the results (TaqMan gene expression assay 4319413E).

To check for the presence of RNA in the enhancer and coding region, total RNA was extracted from untreated cells, reverse-transcribed as above, and 5, 10, and 20 ng of reverse transcribed products were amplified with primers corresponding to different sections of the enhancer and coding region of uPA.
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FIGURE 1. Both uPA enhancer and MP sequences are present in genomic DNA immunoprecipitated with either anti-Sp1 or anti-p300 antibodies. ChIP-ready chromatin was immunoprecipitated with anti-Sp1 and anti-p300 polyclonal antibodies. Purified DNA was amplified by PCR with the F5/R14, F27/R31, and F14/R26 primer sets (Table 1). The PCR products were fractionated on a 2% agarose 0.5 × TBE gels and stained with EtBr.

A scheme of the regulatory region of the uPA gene (E, enhancer; MP, minimal promoter) and location of the primer sets (not drawn to scale). The regulatory elements are located ~2 kb apart. Empty arrows, transcription start site. Empty boxes, amplified fragments.

PCR amplification products of immunoprecipitated genomic DNA. Both enhancer and MP sequences, but not the intervening sequence, are present in the material recovered after immunoprecipitation with anti-Sp1 or anti-p300 antibodies.

For ChIP assays, cross-linked material was digested for 50 min at 37 °C, and MNase digestion was stopped by adding radiolabeled material at each time point and directly added to the digestion stop solution (1% SDS, 0.1 M NaCl, 10 mM EDTA, 10 mM EGTA, 50 mM Tris-HCl, pH 8). Digested chromatin was treated with proteinase K (500 µg/ml) for 5 h at 37 °C, cross-links were reverted by heating overnight at 65 °C, and the DNA was purified using phenol extraction and resuspended in distilled water. The DNA was then treated with RNase A (50 µg/ml) for 30 min at 37 °C, again with proteinase K (500 µg/ml) for 5 h at 55 °C, phenol-extracted, and precipitated. For the experiments shown in Figs. 2–4 and 6, the DNA was resuspended in distilled water and quantitated at the spectrophotometer (A₂₆₀), and equal amounts (100 ng) of material, for each time point, were used as template in PCR reactions.

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ChIP—Each aliquot of MNase-digested cross-linked chromatin (200 µg) was preclotted with 25 µl of Protein A-Sepharose beads (Amersham Biosciences), previously coated with 10 µg/ml each of poly(dl-dc), poly(dG-dc), and poly(dA-dT) and with 100 µg/ml of bovine serum albumin in radioimmune precipitation assay buffer. The aliquots were then incubated overnight with 1 µg of the appropriate antibodies (or without antibodies for the mock controls) in a total volume of 1 ml of radioimmune precipitation assay buffer and immunoprecipitated as described (27). Following immunoprecipitation, the material was treated with RNase A (50 µg/ml) for 30 min at 37 °C and by proteinase K (500 µg/ml) in 0.5% SDS at the same temperature overnight. Formaldehyde cross-links were reverted by heating the samples at 65 °C for 5 h, and the DNA was purified with phenol extraction and then resuspended in 250 µl of distilled water. Resuspended material (4 µl) was used as a template in PCR reactions.

PCRs were performed as follows: (a) first denaturation: 95 °C for 3 min; second denaturation: 95 °C, 1 min; annealing step (see Table 1 for temperatures of the primer sets), 1 min; extension: 72 °C, 1 min; final extension: 72 °C, 3 min. The second denaturation, annealing, and extension steps were normally repeated for 33 cycles. To exclude the presence of a signal from other immunoprecipitated material, the number of cycles in the PCR reactions was raised to 40. Primer sequences, location with respect to the uPA sequence (17), and annealing temperatures for each primer set are reported in Table 1. For the amplification of the 1574-bp genomic fragment shown in Fig. 1 (primers F14/R26) an elongation step of 2 min was used. PCR products were analyzed on 2% agarose gels in 0.5 × TBE buffer. (45 mM Tris-borate; 1 mM EDTA, pH 7)

The specific antibodies used for immunoprecipitations were against the following modifications of histone H3: K4me2 or K9me2 (#07–030 or #07–352, respectively, Upstate Biotechnology); K14ac or K9ac (#07–353 or #07–352, respectively, Upstate Biotechnology). The nomenclature of histone H3 post-translational modifications is according to a previous study (28). Anti-HMGN1 and -HMGN2 antibodies were a kind gift of Dr. Michael Bustin, NCI, National Institutes of Health, Bethesda, MD. Polyclonal antibodies against c-Jun (sc-1694X, Santa Cruz Biotechnology, Santa Cruz, CA) and monoclonal antibodies against CTD-P-S2 (MMS-129-RA, Covance) and CTD-P-S2 (MMS-134-RA, Covance) were also used. In all experiments, polyclonal antibodies against the uPA receptor produced in our laboratory were used as unrelated antibodies. In all the experiments investigating the protein composition of MNase-digested chromatin, material from the same immunoprecipitation was amplified with primers located in the enhancer and minimal promoter regions as indicated in the individual figures.

RESULTS

Anti-Sp1 or -p300 Antibodies Immunoprecipitate the uPA MP and Enhancer Sequences—Formaldehyde cross-links molecules with reactive groups at a maximum distance of 2 Å. This
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FIGURE 2. MNase digestion time course of ChIP-ready chromatin and detection of nucleosome-size fragments in the enhancer region. A, 1,200 μg of ChIP-ready chromatin were digested with MNase. At increasing times, aliquots were withdrawn, the digestion was stopped, and genomic DNA was purified. After purification, equal amounts of genomic DNA for each time point were fractionated on a 2% agarose gel in 0.5 × TBE and visualized with EtBr. M, markers; left marker, 1-kb DNA ladder (Fermentas); right marker, Mass Ruler DNA Ladder (100-bp ladder; Fermentas). n, 2n, etc. = mononucleosomes, dinucleosomes, etc. B, scheme (not to scale) of the amplified region using the indicated primer sets (Table 1). Striped boxes, amplified fragments; arrows, primers and their orientation. White box with E: enhancer. C, equal amounts of genomic DNA from the different time points (panel A) were amplified by PCR. Only mononucleosome-size fragments (145–189 bp) can be amplified in the enhancer region using genomic DNA from all digestion time points.

FIGURE 3. Detection and characterization of DAF amplicons in the MP region of the uPA gene. A, scheme (not to scale) of the amplified regions and primers used. Full arrowheads, primers (Table 1) and their orientation. Empty arrowhead, transcription start site. White and hatched boxes, amplified fragments; MP, minimal promoter; I, first, untranslated exon of the uPA gene; white and black boxes with I: untranslated and translated portions, respectively, of the second exon of the uPA gene. B, equal amounts of genomic DNA from each MNase digestion time point were amplified with the appropriate primers (panel A), and PCR products revealed on a 2% agarose gel in 0.5 × TBE stained with EtBr. Amplicons F22/R26 and F26/R31 show a loss of PCR signal using primers F8/R11 and F8/R12, respectively. A loss of PCR signal is also seen using primers F8/R14 and F8/R11. C, equal amounts of genomic DNA from the MP region using the indicated primer sets (Table 1). Striped boxes, amplified fragments; arrows, primers and their orientation. White box with E: enhancer. The signal is then recovered at later time points revealing the presence of amplicons resistant to MNase digestion. C, amplification products of the MP region using the F22/R31 primer set were fractionated and visualized as above. Amplification products are visible only at the 0- and 5-min time points, indicating that amplicons F22/R26 and F26/R31 do not belong to the same genomic fragment but represent different chromatin populations.

may occur between proteins bound to distant regulatory elements, if such groups are close enough, implying their interaction. We tested this hypothesis for the MP and the enhancer and the cleavage by MNase of the uPA enhancer region.

PCR Reactions with Genomic DNA from MNase-digested ChIP-ready Chromatin Reveal Fragments with a Distinctive

of the uPA gene, located 2 kb upstream, by performing conventional ChIP experiments on cross-linked, sonicated chromatin from PC3 cells with antibodies against Sp1, which uniquely binds the MP (18, 21, 22, 25), and the cofactor p300. We asked if the DNA immunoprecipitated with one or the other antibody contained both enhancer and MP sequences, by amplifying the recovered material with specific primers (F5/R14 and F27/R31 in Fig. 1A). Indeed both genomic DNA sequences were immunoprecipitated with either antibody, whereas the IVS was not detected (Fig. 1B) even by increasing the number of PCR cycles. Thus, the results are consistent with a close physical proximity of the regulatory elements and the extrusion of the IVS.

However, amplification of the input DNA with the F14/R26 primer set did show the presence of a PCR product (Fig. 1B), indicating that the IVS was not fully broken by sonication. To obtain independent evidence of the interaction of the two separate genomic fragments we enzymatically cleaved chromatin prior to immunoprecipitation.

ChIP-ready Chromatin Is Accessible to MNase Cleavage—We chose to digest ChIP-ready chromatin with MNase, a processive enzyme widely employed in chromatin studies (29, 30). The pattern of MNase digestion obtained in a time-course experiment shows that ChIP-ready chromatin from PC3 cells was readily and increasingly cleaved by the enzyme to poly-, mono-, and sub-nucleosomal particles (Fig. 2A). Next, genomic DNA from the various digestion time-points of Fig. 2A was subjected to PCR reactions with sets of primers that amplified increasingly larger (from 145 to 250 bp) fragments in the uPA enhancer region (Fig. 2B). The results showed that only nucleosome-size genomic fragments could be amplified using material from all the digestion time points (Fig. 2C), thus confirming the accessibility to


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Amplification Pattern in the MP Region—We then repeated the PCR amplification using primer sets in the uPA MP region. Because we wanted to exclude the presence of genomic fragments larger than 200 bp (mononucleosome size), we deliberately designed two sets of primers spanning 464 bp and 320 bp, respectively, and expected to be able to amplify genomic DNA only by using material from the early digestion time points (see Fig. 2A).

As shown in Fig. 3B, primer sets F22/R26 and F26/R31 showed that the amplification signal decreased by using genomic DNA from early and intermediate MNase digestion time points but was rescued with material from later (20 and 50 min) time points. However, the amplification of two fragments of similar size upstream of F22 and one in the enhancer region displayed the progressive loss of the PCR signal, as expected (data not shown). Amplicons F22/R26 and F26/R31 share a common primer (F/R26 in Fig. 3A); however, they displayed the lowest amplification signal at different time points. We asked if they belonged to the same or different genomic populations by amplifying the region spanning both fragments (using primers F22 and R31 in Fig. 3A). In this case the amplification signal decreased with increasing digestion time (Fig. 3C). Thus amplicons F22/R26 and F26/R31, specifically located at and upstream of the MP region of the uPA gene, belong to different DNA fragments. Because F22/R26 and F26/R31 amplicons have a distinctive amplification pattern they were named DAF-A and DAF-B, respectively.

The amplification pattern of DAF-A and -B apparently contradicts the MNase digestion kinetics and the results obtained in the enhancer region, shown in Fig. 2. However, one must recall that MNase is a processive endonuclease (30), and this mode of action has two main consequences: 1) it causes a progressive and generalized loss of genomic DNA, as indicated by the quantitation of the material recovered at each MNase digestion time point (supplemental Fig. S1C). 2) It enriches genomic fragments that originate from a chromatin population that is more resistant to MNase cleavage than bulk chromatin, whereas it rapidly degrades those from a chromatin population more sensitive to cleavage than bulk chromatin. We therefore speculated that DAF-A, -B, and -Bx originate from such resistant populations. Thus we performed Southern blot experiments to test this point. Overall the results (shown in supplemental Fig. S1) indicated that: 1) both the enhancer and DAF-A regions are more sensitive than bulk chromatin to severing treatments; 2) at early digestion time points mono- and subnucleosomes from DAF-A accumulate more slowly than those form the enhancer region; and 3) DAF-A can only be revealed by the amplification provided by PCR and not by probing a Southern blot. The two latter observations indicate that DAF-A originates from a small, severing-resistant subpopulation of chromatin of the uPA regulatory region.

**Defining the Borders of DAF-A and DAF-B**—Because DAF-A and DAF-B represent different chromatin populations we next established their size by amplifying increasingly longer fragments, assuming that the amplification pattern of amplicons F22/R26 and F26/R31 would be maintained if longer fragments belonged to the same chromatin populations. Indeed we could extend the amplification pattern of DAF-B in the 3’ direction (Fig. 4B), using primers R34 and R36, but not R37, in combination with F26. However, by using primer F25 in combination with R31 the pattern of amplicon DAF-B was lost in the 5’ direction (Fig. 4B). Thus DAF-B could be extended to the untranslated portion of the second exon of the uPA gene (Fig. 4A and Ref. 31). Amplicon F26/R36 was named DAF-B.
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FIGURE 5. Differential protein content of DAF-A, -B, and -Bx. ChIP-ready chromatin was digested for 50 min with MNase and subsequently immunoprecipitated with the antibodies indicated in the figure, with an antibody against the uPA receptor (unrelated) or treated like the immunoprecipitated samples, but omitting the antibody (mock). The immunoprecipitated, purified genomic DNA was amplified with the primer sets specific for DAF-A, -B, and -Bx and fractionated on a 2% agarose gel. Input DNA was a 1:1000 dilution of the DNA from the 50-min digestion time point prior to immunoprecipitation. Symbols are as in Fig. 4. B, amplification of immunoprecipitated genomic DNA with antibodies to histone H3 post-translational modifications and HMGN proteins and C, with antibodies against the functionally different forms of RNAPII. DAF-A, -B, and -Bx display substantially different protein contents, and only DAF-B is associated with the elongating form of RNAPII (CTD-P-S2) indicating the presence of three distinct chromatin population in the uPA MP region.

“extended” (DAF-Bx). Fig. 4C shows that the amplification pattern of DAF-A was lost using either primer F21 (5’ extension) in combination with R26 or primer R27 (3’ extension) in combination with F22. This suggests that primers F22 and R26 are located close to or at the borders of DAF-A.

**DAF-A, -B, and -Bx Amplicons Represent Discrete Chromatin Structures with Different Protein Contents**—Given their proximity to the uPA MP, we asked if the DAF-A, -B, and -Bx amplicons were associated with transcription and analyzed their protein content. We focused our attention on three polypeptides: histone H3, because its post-translational modifications are strictly related to the transcriptionally active or inactive state of the gene (32), HMGN proteins, which are components of active chromatin (33), and RNAPII. To perform the experiments, ChIP-ready chromatin was digested with MNase for 50 min (and yielded only mononucleosome-size genomic fragments; supplemental Fig. S2) and subsequently immunoprecipitated with the antibodies indicated in Fig. 5. The resulting genomic DNA was amplified by PCR using the primers sets for DAF-A, -B, and -Bx (Fig. 5A).

The results in Fig. 5B show that the histone modifications associated with DAF-B (H3K4me2, H3K9ac, and H3K14ac) are all established marks of transcriptionally active chromatin (34, 35) (Fig. 5B). This is in agreement with our previous results showing the presence of a DNase I-hypersensitive site on the form of RNAPII prompted the speculation that the amplicons might represent large complexes of transcriptional nature. Thus we treated PC3 cells with α-amanitin, an inhibitor of RNAPII (26, 37), and asked what was the fate of the DAF amplicons in drug-treated PC3 cells, assuming that complexes not involved in transcription would persist despite the treatment. ChIP-ready chromatin was prepared from α-amanitin-treated cells and then used for the MNase digestion time-course experiment in which bulk chromatin yielded a digestion pattern similar to that of untreated cells (Fig. 6B, compare with Fig. 2A). Purified DNA from each digestion time point was amplified with the specific primers for DAF-A, -B, and -Bx and with the F29/R31 primer set, amplifying a nucleosome-size fragment (199 bp) within DAF-B (Fig. 6A). Fig. 6C shows that the PCR signals of DAF-A, -B, and -Bx gradually decreased and were not rescued by using material from the 50-min time point, unlike the experiments in Fig. 3B. However, at all digestion time points we were still able to detect nucleosome size genomic fragments located within DAF-B (199 bp in Fig. 6C) on the enhancer (150 bp, not shown), suggesting that the drug treatment had perturbed the specific structures associated with DAF-A, -B, and -Bx, but not the overall structure of the regulatory region. The results indicate that the presence of the DAF amplicons depends on active transcription.

uPA MP in PC3 cells (36). We also found that DAF-B is associated with HMGN1, another hallmark of transcriptionally active chromatin. Unexpectedly, we found DAF-Bx to be associated only with H3K9ac and H3K14ac (Fig. 5B), which suggests that DAF-B and -Bx may represent different chromatin populations. This view is supported by the experiment showing that only DAF-B is associated with RNAPII in its elongating form (phosphorylated at serine 2 of the CTD), whereas DAF-A and DAF-Bx are not (Fig. 5C). Finally, DAF-A contained histone H3 both acetylated and dimethylated at lysine 9. This finding is intriguing, because the modifications affect the same residue and have opposite effect on transcriptional activation, but we did not investigate this issue further. Overall the results indicate that the uPA MP region contains three distinct chromatin populations, one of which (DAF-B) is actively engaged in transcription.

**Presence of DAF-A, -B, and -Bx Depends on Ongoing Transcription**—The protein composition of DAF-A, -B, and -Bx and the specific association of DAF-B with the elongating
Because DAF-B spans the MP and was found associated with RNAP II, it appeared the most likely candidate for such an interaction. Therefore we asked if the immunoprecipitates that contained DAF-B (see Fig. 5) also included one or more fragments (~145 bp to ~190 bp long) encompassing the uPA enhancer, as detailed in the scheme of Fig. 7A. The results of Fig. 7B show that all the immunoprecipitates contained fragments spanning the enhancer, with the notable exceptions of fragment 6, which was not immunoprecipitated by any of the antibodies used (Fig. 7B). However, the only fragment immunoprecipitated by the same combination of antibodies that also immunoprecipitated DAF-B, including α-CTD-P-S2 and α-HMG1, was fragment 1 (Fig. 7B). This indicates that fragment 1 and DAF-B represent populations of enhancer and MP interacting with each other.

**Presence of RNAP II on the uPA Enhancer Is due to Its Interaction with the MP**—The specific presence of RNAP II on the uPA enhancer was also supported by the results shown in Fig. 7B, in which fragment 8 (immediately downstream of fragment 1) is devoid of either form of RNAP II. These findings raise the possibility that the enhancer region is part of an independent transcription unit, nested in the uPA locus. To formally exclude the presence of RNA in the enhancer region, we reverse transcribed total RNA from PC3 cells with random primers and amplified the resulting products with primers spanning fragment 1 and fragments of similar size located 5’ (fragment 7) or 3’ (fragment 8) of fragment 1 (Fig. 8A); we also amplified a fragment from +194 to +420 of the coding region of the uPA gene as positive control. The results in Fig. 8B clearly indicate that the enhancer and neighboring regions are devoid of mRNAs, which, as expected, is present in the coding region of the uPA gene. Therefore, the presence of RNAP II CTD-P-S2 on the enhancer is likely due to its interaction with the MP. This was further supported by the absence of fragment 1 in the

**Chromatin Proteins and RNAP II Content of Nucleosome-size Fragments in the uPA Enhancer Region**—We thought that the presence of specific transcription-dependent chromatin structures in the uPA MP region might facilitate our search for evidences of an interaction between the enhancer and the promoter. The concentration of α-amanitin to be used in PC3 cells treatment (10 μg/ml for 24 h) was determined in a dose-response experiment in which the endogenous levels of uPA mRNA were determined by quantitative reverse transcription-PCR at each α-amanitin concentration (data not shown). A, the scheme of the amplified regions and the primers used for DAF amplicons are as in Fig. 4. Squared box, the 199-bp (nucleosome size) fragment amplified with the F29/R31 primer set is contained in the DAF-B amplicon. B, MNase digestion of cross-linked chromatin from α-amanitin-treated PC3 cells generates a genomic DNA ladder similar to that obtained from untreated cells (compare with Fig. 2A). n, 2n, and 3n: mono-, di-, and trinucleosome size DNA. M, DNA size marker (fragments length is indicated to the right). C, loss of the amplification pattern of DAF-A, -B, and -Bx using MNase-digested, ChIP-ready chromatin from α-amanitin-treated PC3 cells, as detected on a 2% agarose, 0.5 TBE gel stained with EtBr. The results indicate that the structures associated with the DAF amplicons are sensitive to drug treatment of PC3 cells, but the nucleosomal structure of the uPA regulatory region is maintained.
**MNase-resistant Amplicons in the uPA Promoter**

**FIGURE 8.** The presence of RNAPII on the uPA enhancer is not due to a nested transcription unit and is sensitive to α-amanitin treatment of PC3 cells. A, scheme depicting the primer sets used and the amplified fragments in the enhancer region of the uPA gene is not to scale. Block box with E, enhancer. B, total RNA was extracted and reverse-transcribed, and increasing amounts were amplified with the primers indicated in the figure, spanning fragments 1, 7, and 8 in the enhancer region and a 226-nt fragment in the coding region (see Table 1). As controls, genomic DNA and non-retrotranscribed RNA were also amplified with the same primers. PCR products were visualized on a 2% agarose, 0.5× TBE gel stained with EtBr. The lack of mRNA in this region indicates the absence of a cryptic transcription unit. C, MNase-digested (50 min), ChIP-ready chromatin from α-amanitin-treated PC3 cells was immunoprecipitated with antibodies against the phosphorylated forms (CTD-P-S2 and CTD-P-S5) of RNAPII (uPA Enhancer, Is Also Associated with the DAF-B Amplicon).—To reciprocate the experiment of Fig. 1, we asked whether antibodies against c-Jun, a transcription factor that specifically binds the uPA enhancer and not the MP (21-25) could also immunoprecipitate DAF-B. Immunoprecipitated material from MNase-digested (50 min) ChIP-ready chromatin was amplified with primer sets for fragments 1, 7, and 8 on the enhancer and DAF-B on the MP (F7/R10, F11/R15, and F26/R31, respectively, Fig. 9A). The results showed that both fragment 1 and DAF-B sequences were present in the immunoprecipitate (Fig. 9B). However fragment 8 (immediately downstream of fragment 1) is devoid of c-Jun (Fig. 9B). Furthermore the presence of c-Jun in the enhancer and MP regions was abolished by the α-amanitin treatment of PC3 cells (Fig. 9C), further corroborating the role of these fragments in the interaction. Overall, our results lead us to conclude that fragment 1 and DAF-B are populations of interacting enhancer and MP.

**DISCUSSION**

In this report we provide evidence that the interaction between the enhancer and the MP of the uPA gene occurs through specific structures, identified and characterized through their sequences and protein composition. In particular, the sequences at and around the MP region were discriminated by their distinctive and persistent amplification.

immunoprecipitate (with a-CTD-P-S5 and a-CTD-P-S2) of MNase-digested, ChIP-ready chromatin obtained from α-amanitin-treated PC3 cells (Fig. 8C), condition in which we observed the loss of DAF-B (Fig. 6B).
pattern, after extensive MNase digestion of ChIP-ready chromatin. The results support a model in which the interaction of enhancer and MP is transcription-dependent, persists during the early stages of transcriptional elongation, and may cause the extrusion of the intervening sequence (looping). Besides the presence of the same chromatin proteins (HMGN1) and post-translational modifications of histone H3 (H3K4me2, H3K9ac, and H3K14ac), the interaction of uPA enhancer and MP is supported by the association of specific genomic fragments (DAF-B and fragment 1), spanning the regulatory elements, with the elongating form of RNAP II and with transcription factors that specifically bind either the enhancer (c-Jun) or the MP (Sp1) (Figs. 1 and 9). Indeed, p300 may bridge these two proteins contributing to the interaction between enhancer and MP (38–40). Interestingly, the association of the same histone H3 modifications and chromatin proteins with both the uPA enhancer and MP does not determine whether these proteins are actually bound on both elements or if they are differentially contributed by enhancer and MP to the final structure. From a functional standpoint, such a distinction is irrelevant, because both elements (and related chromatin structures) are required for transcriptional activation. More importantly, this is further supported by the association of RNAP II, Sp1, and c-Jun with both elements, regardless of the location of their specific binding site, indicating that the resulting structure functions as a single transcription control unit, as shown in the model of Fig. 9D. Interestingly, one of the sequences embedded in this unit (DAF-B) represents actively transcribing chromatin, given its association with the elongating form of RNAP II, and spans from –205 to +114, thus including a substantial portion of the coding region. This observation suggests that the interaction between enhancer and MP is maintained during the early stages of transcriptional elongation.

What generates the complexes that give rise to MNase-resistant genomic fragments? It is unlikely that this is due to nucleosomes sliding in a closely packed configuration because of the presence of a DNase I-hypersensitive site in PC3 cells in the exact location of the DAF-B amplicon (18). We favor the hypothesis that DAF amplicons are transcriptionally competent complexes. Indeed, DAFs become undetectable after α-amanitin treatment of PC3 cells, whereas the phosphorylated form of RNAP II is bound to DAF-B. Both evidences imply that ongoing transcription is a requisite for their presence. Nevertheless, DAF-A, -B, and -Bx amplicons appear to underlie different chromatin populations, as indicated by their physical boundaries and protein content (see Figs. 4 and 5).

DAF amplicons represent chromatin subpopulations of all the copies of the gene present in ChIP-ready material before MNase digestion. Their amplification pattern becomes visible only when the sensitive population is fully digested, and this occurs only at long digestion time points, when a large amount of material has been cleaved to mononucleosomes (see Fig. 2 and supplemental Fig. S1). Although we do not know how many copies of the gene are transcribed per cell at any given time, both observations are consistent with previous reports indicating that constitutively active genes are not continuously transcribed. They shuttle between “on” and “off” states spending more time in the “off” state, so that, at any given time (steady-state level), the number of copies that are effectively engaged in transcription is underrepresented (41–43). Furthermore, the steady-state uPA mRNA levels in PC3 cells indicate that not all gene copies are transcribed (19). It is therefore possible that DAF-A, -B, and -Bx represent different structures formed during the steps of transcription onset occurring at low kinetic rate (44), such as promoter escape by RNAP II (45, 46).

Concerning the technique employed, the β-globin cluster has provided a paradigm for long distance interactions of regulatory elements. Chromosome configuration capture experiments (11) have lead to the identification of interacting sequences (12, 14), whereas independent ChIP experiments have characterized the histone content of the chromatin fiber of the cluster (47, 48). In this system, therefore, the protein composition of the interacting sequences has been inferred, and formal evidence that specifically interacting structures have a defined protein composition is not yet available. Extensive digestion of ChIP-ready chromatin with MNase, on the other hand, generates genomic fragments that are physically defined by the end point of cleavage, because MNase is a processive enzyme that stops when it finds (a physical) barrier that hinders further cleavage. Moreover, the large fragments represent a small subpopulation of all those generated by extensive digestion (mostly mono- and sub-nucleosomal in size) and dramatically reduces the possibilities of detecting false-positive results in ChIP experiments.

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