Mechanism of Nucleosome Dissociation Produced by Transcription Elongation in a Short Chromatin Template[†,§]

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Received December 15, 1994; Revised Manuscript Received February 22, 1995[⊗]

ABSTRACT: We have used a linear DNA template (239 bp) containing a nucleosome positioning sequence (NX1) downstream of the T7 RNA polymerase promoter to study the mechanism of transcription elongation through a nucleosome. Under ionic strength approaching physiological conditions we have observed that transcription causes nucleosome dissociation and histone redistribution within the template. We have examined the role of the different elements that, in principle, could induce nucleosome dissociation during transcription. The high affinity of histones for single-stranded DNA observed in titration experiments performed using the purified (+) and (−) strands of the NX1 fragment suggests that nucleosome dissociation is not due to the formation of segments of single-stranded DNA by RNA polymerase in the elongation process. Furthermore, our results show that although RNA can interact with core histones, the synthesized RNA is not bound to the histones dissociated by transcription. Our results indicate that core histones released during transcription can be bound to naked DNA and chromatin (with or without histones H1−H5). From the dynamic properties of excess histones bound to chromatin, we suggest a nucleosome transcription mechanism in which displaced histones are transiently bound to chromatin and finally are reassembled with DNA after the passage of the polymerase.

Nucleosomes are present even in chromatin regions where replication and transcription are taking place (Sogo et al., 1986; DeBernardin et al., 1986). In spite of the detailed knowledge of the three-dimensional structure of both the nucleosome (Struck et al., 1992) and the core histone octamer (Arents & Moudrianakis, 1993), little is known about the mechanisms by which nucleosomes participate in these fundamental cellular functions. It seems clear that the precise positioning of nucleosomes in particular DNA sequences and their interaction with transcription factors [reviewed in Simpson (1991) and Workman and Buchman (1993)] is directly related to the regulation of chromatin activity, but the knowledge of the molecular mechanisms that allow the elongation of RNA chains in eukaryotic genes containing nucleosomes is very limited.

The linker histones H1 and H5 are partially displaced from active genes (Kamakaka & Thomas, 1990; Postnikow et al., 1991). Core histones associated with promoters block initiation of transcription (Wolffe & Drew, 1989; Kirov et al., 1992). However, the results of different laboratories have suggested various mechanisms for transcription elongation [reviewed in Thoma (1991), Morse (1992) and van Holde et al. (1992)].

Losa and Brown (1987) have observed that SP6 RNA polymerase can transcribe through nucleosome core particles in 5S ribosomal genes without displacing histones. On the other hand, the in vivo studies of Jackson (1990) have shown that the bulk of transcription in MSB cells releases a large fraction of H2A,H2B dimers, whereas H3,H4 tetramers remain bound to DNA. Lorch et al. (1988) have found that in general SP6 RNA polymerase causes the dissociation of nucleosome cores during transcription, and Karpov et al. (1984) have reported that core histones are released from actively transcribed heat-shock *Drosophila* genes, but these genes are not depleted of histones when transcription occurs at lower rates. From studies performed using cross-linked histones, O'Neill et al. (1993) have suggested that the histone octamer as a unit may be displaced by RNA polymerase from its original binding site while maintaining a weakened interaction with DNA. More recently, Studitsky et al. (1994) have shown that at low transcription rates histones are translocated from their original binding site without leaving the template, but at higher transcription rates nucleosome dissociation occurs on a short DNA template containing an SP6 RNA polymerase promoter. Histone redistribution and displacement during transcription have also been reported by O'Donohue et al. (1994).

Which are the mechanisms that in some chromatin transcription systems produce the dissociation of histones from DNA? Nucleosome dissociation could be produced either by the direct contact of the polymerase molecule with the nucleosome core or indirectly by the DNA topological stress generated during transcription (Liu & Wang, 1987; Pfaffle & Jackson, 1990; Lee & Garrard, 1991; Clark & Felsenfeld, 1992). Furthermore, since the normal structure of the nucleosome containing double-stranded DNA (ds-DNA)[1] must be strongly perturbed to allow the formation of single-stranded DNA (ssDNA) segments during transcription (Gamper & Hearst, 1982), it can be suggested that the

[†] This work was supported in part by DGICYT grants PB89-0305 and PB92-0602. F.G. is a recipient of a predoctoral fellowship from the Ministerio de Educación y Ciencia. X.F.-B. was supported by a predoctoral fellowship from the Generalitat de Catalunya.

[§] The DNA sequence reported in this paper has been submitted to the EMBL Nucleotide Sequence Database under Accession Number X69506.

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[⊗] Abstract published in *Advance ACS Abstracts,* May 1, 1995.

0006-2960/95/0434-6711$09.00/0

formation of ssDNA may induce nucleosome dissociation. Finally, naked DNA or other negatively charged molecules such as RNA and nucleosomes present in the in vitro transcription systems may also promote histone displacement during transcription. Taking into account all these considerations, in this work, we have investigated the mechanism that produces the nucleosome dissociation that we have observed in the transcription of a small chromatin template containing a T7 RNA polymerase promoter and a nucleosome positioning sequence. Although T7 RNA polymerase is a prokaryotic enzyme, the results of Benton et al. (1990) showing that this enzyme can transcribe in vivo specific genes in yeast cells strongly suggest that the mechanism of transcription elongation proposed from the results obtained using this enzyme is probably the same for eukaryotic RNA polymerases.

MATERIALS AND METHODS

Preparation of Nucleosomes, Histones, Chromatin Fragments, and M13 DNA. Core histones, histones H2A,H2B and H3,H4, and nucleosome core particles were obtained from chicken erythrocyte nuclei as previously described (Aragay et al., 1988; Samsó & Daban, 1993). Chromatin fragments (~10 nucleosomes) depleted of histones H1−H5 were prepared as described (Aragay et al., 1991). Native chromatin fragments (~30 nucleosomes) containing H1−H5 were obtained and characterized following procedures described elsewhere (Bartolomé et al., 1994). The histone composition of chromatin samples was analyzed on sodium dodecyl sulfate−polyacrylamide gels stained with the fluorescent dye Nile red (Daban et al., 1991). Circular dsDNA and ssDNA from bacteriophage M13 were isolated and analyzed by conventional procedures (Messing, 1983).

Cloning and Isolation of the Core NX1 DNA Fragment: Purification of the (+) and (−) Strands. Chicken erythrocyte core particles were deproteinized by treatment with proteinase K followed by phenol extraction. The resulting DNA was treated with T4 polynucleotide kinase and ATP, blunt-end ligated into the *Sma*I site of phosphatase-treated M13mp9, and cloned in *Escherichia coli* JM103 (Messing, 1983). A randomly chosen clone containing the DNA insert corresponding to one nucleosome (named NX1 in this work) was sequenced according to the method of Sanger et al. (1980) using a Pharmacia−LKB automated laser fluorescent sequencer. The NX1 DNA insert was excised from the M13 vector by digestion with *Eco*RI and *Bam*HI and further amplified using a Bluescript (Stratagene) vector. Finally, *Eco*RI and *Bam*HI were used to remove the insert from this plasmid. The linear plasmid was separated from the NX1 fragment by precipitation with 7.5% poly(ethylene glycol) in 0.55 M NaCl, 1 mM EDTA, and 10 mM Tris-HCl, pH 7.5. The resulting NX1 DNA was phenol-extracted twice, precipitated with 2-propanol, and washed with ethanol. The (+) and (−) strands of NX1 DNA were purified by poly(ethylene glycol) precipitation as described elsewhere (Fernandez-Busquets & Daban, 1992).

Preparation of the DNA Template. *Bss*HII and *Bam*HI were used to remove from the Bluescript vector a 239-bp DNA fragment (named NX1P7 in this work) containing the promoter for T7 RNA polymerase and the NX1 sequence. In order to prepare large amounts of the NX1P7 fragment, it was selectively precipitated with 7% poly(ethylene glycol) and, when necessary, further purified by HPLC on an anion-exchange Gen-Pak Fax (Waters) column (4.6 × 100 mm). The gradient used was 0.45−0.60 M NaCl (linear) in 25 mM Tris-HCl and 1 mM EDTA, pH 8.0.

Reconstitution Procedures and DNase I Footprinting. Nucleosomes were reconstituted onto NX1P7 DNA (~75 μg/mL) by salt-gradient dialysis or direct mixing in 50 mM NaCl, 40 mM Tris-HCl, and 1 mM EDTA, pH 8.0, following procedures previously described (Daban & Cantor, 1982). Samples for footprinting analysis were digested with DNase I (Boehringer) and electrophoresced on a nondenaturing 6% polyacrylamide gel. The resulting bands were excised from the gel, and the DNA was extracted by electroelution for 2 h at 4 V/cm in 0.1 × TBE buffer (9 mM Tris−borate and 0.25 mM EDTA, pH 8.3) containing 0.1% SDS. The electroeluted DNA was purified with phenol, precipitated and washed with ethanol, and loaded onto a 6% polyacrylamide gel containing 7 M urea. After electrophoresis for 3 h at 1800 V, the resulting bands were transferred to a nylon membrane, fixed by UV irradiation, and hybridized with a biotin-labeled probe (23 nucleotides) complementary to the T7 promoter. The DNA bands were detected using alkaline phosphatase and a dioxetane chemiluminescent substrate (Millipore). This chemiluminescent method was also used to detect the bands of the dideoxy sequencing reactions with T7 DNA polymerase (Pharmacia) carried out to locate the DNase I cleavage sites.

Transcription Reactions. DNA and reconstituted chromatin templates (~63 μg of DNA/mL; 0.5−1 μg of DNA) were transcribed generally at 37 °C for 30−90 min with T7 RNA polymerase (Pharmacia; 40 units/μg of DNA) in the presence of 50 mM NaCl, 40 mM Tris-HCl (pH 8.0), 1.7−8 mM $MgCl_2$, 0.5−1 mM spermidine, 24 mM DTT, RNase inhibitor (Boehringer; 25 units/μg of DNA), and 0.3 mM each ATP, UTP, GTP, and CTP. The reaction was stopped by the addition of EDTA to 8 mM. Some samples were digested with 0.5 μg of RNase (Boehringer) for 2−3 h at the end of transcription. The chromatin templates, free DNA, and the RNA transcript were analyzed on nondenaturing polyacrylamide gels (16 cm long) containing TBE. The final DNA concentration of the samples loaded onto the gels was 45−50 μg/mL. Electrophoresis was performed for 2.5 h at 250 V. The gel was stained with ethidium bromide and photographed over an ultraviolet light. Photographic negatives were scanned with a Shimadzu CS-9000 densitometer.

Association of Histones with ssDNA. NX1 ssDNA, either in the absence or in the presence of core histones, was digested with micrococcal nuclease (Sigma) in a buffer containing 0.2 M NaCl, 10 mM $CaCl_2$, 1 mM EDTA, and 10 mM Tris-HCl, pH 7.4. The digestion was carried out at room temperature with 0.2 units of enzyme/μg of DNA and stopped at different times by addition of EDTA to a final concentration of 20 mM. The resulting samples were mixed with SDS to 0.03%, loaded onto a 6% polyacrylamide gel containing TBE and 0.03% SDS, and electrophoresed at 100 V for 1 h. In the titration and competitive association experiments, histones were mixed with ssDNA and/or dsDNA in 0.2 M NaCl, 1 mM EDTA, and 10 mM Tris-HCl, pH 7.4, incubated at room temperature for at least 30 min, and finally loaded onto the indicated gels. Samples

[1] Abbreviations: dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; TBE, 0.09 M Tris−borate and 2.5 mM ethylenediaminetetraacetic acid (EDTA), pH 8.3; DTT, dithiothreitol.

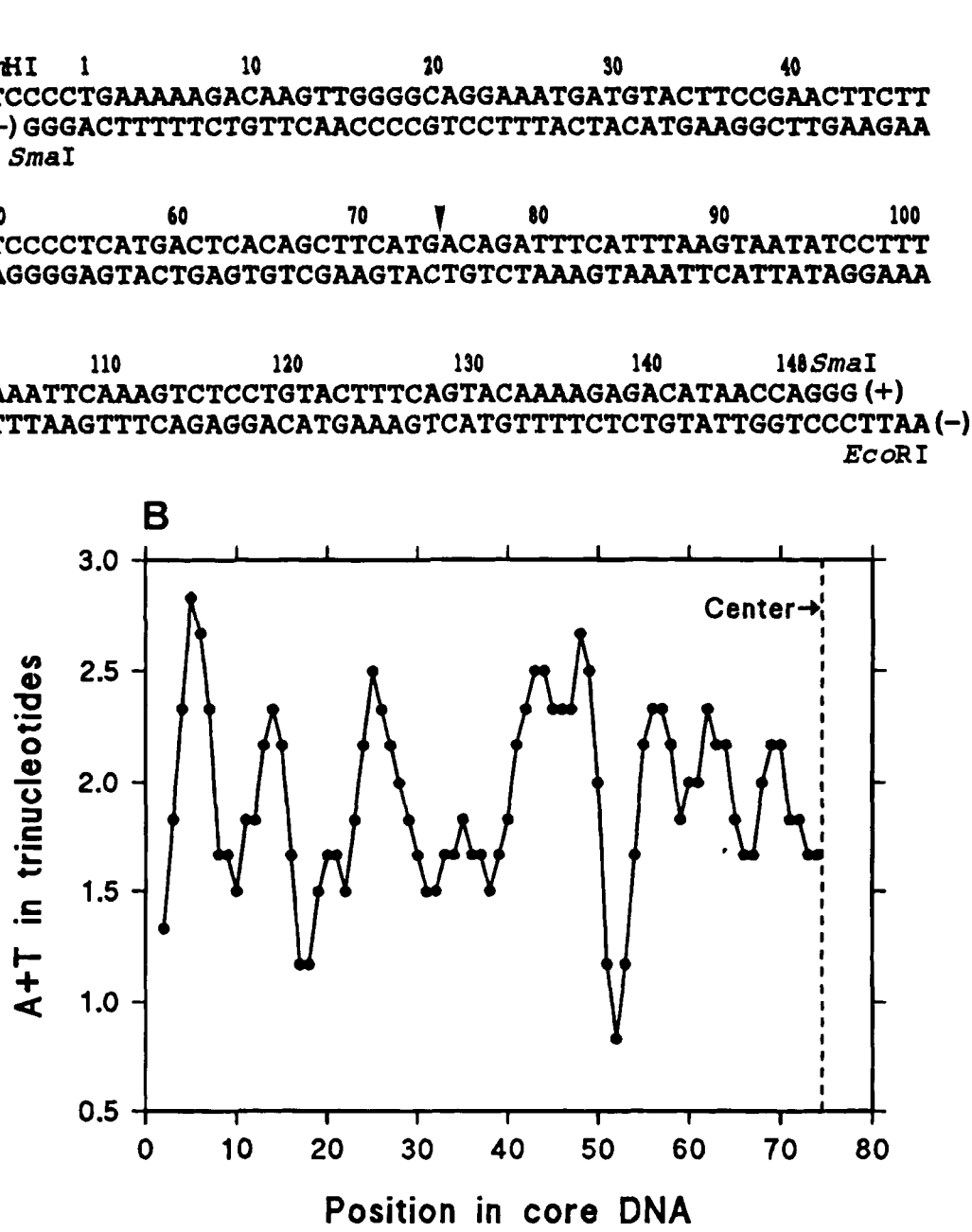

FIGURE 1: Sequence of the NX1 fragment used in this study. (A) In addition to the cloned nucleosome core DNA (positions 1−148), the *Bam*HI, *Sma*I, and *Eco*RI sites of the cloning vector are also indicated; the arrow indicates the center of the sequence. (B) Number of A and T in tracts of 3 nucleotides along the NX1 sequence. The value shown for each position i corresponds to the number of A + T found in the tract $(i - 1) - i - (i + 1)$ averaged about the center of the sequence (i.e., position 74.5).

containing low molecular weight DNA were analyzed on nondenaturing 6% polyacrylamide gels (15 cm long) containing TBE buffer as described previously (Aragay et al., 1988, 1991). Samples containing M13 DNA were electrophoresed on nondenaturing 1.4% agarose gels (25 cm long) containing TBE.

RESULTS

Nucleosome Positioning in the Template. The sequence of the cloned DNA (NX1 fragment; 148 bp) from nucleosomal origin used in this study is shown in Figure 1A. The distribution of A/T-rich tracts in this fragment is shown in Figure 1B. The observed periodic modulation is compatible with the frequencies of occurrence of short tracts containing A/T found in many natural nucleosome DNA sequences (Satchwell et al., 1986) and in artificial nucleosome positioning sequences (Shrader & Crothers, 1989). The remarkable similarities between Figure 1B of this work and the periodic modulation of occurrence of dinucleotides and trinucleotides containing A/T detected in a representative population of nucleosome DNA sequences (Satchwell et al., 1986) indicate that the sequence of the NX1 fragment corresponds to that of a typical nucleosome.

The template (NX1P7; 239 bp) used for transcription studies contains the T7 promoter (Jorgensen et al., 1991) located 58 bp from the *Eco*RI end of the NX1 sequence. At a low core histone to NX1P7 DNA weight ratio, a single and well-defined nucleoprotein band (N1 in Figure 2A) is observed on nondenaturing gels. The approximately 10-bp periodicity of the DNase I cutting pattern of this band (Figure

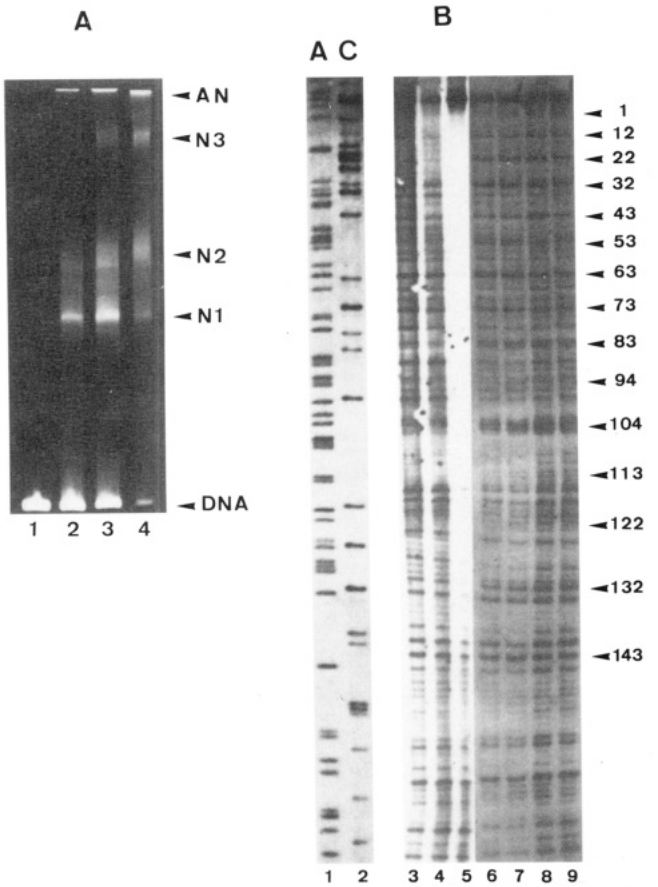

FIGURE 2: Nucleosome positioning in the NX1P7 DNA template. (A) Analysis of core histone−NX1P7 DNA complexes on a nondenaturing gel. The input weight ratios of histones to DNA were (lanes 1−4) 0, 0.3, 0.8, and 1.3, respectively. (B) DNase I footprinting of the chromatin template. The NX1P7 DNA associated with increasing amounts of core histones was digested with DNase I (0.1 unit/μg of DNA; 15 min), electrophoresed on a nondenaturing gel as in (A), and finally, the DNA was extracted from the nucleoprotein bands N1, N2, N3, and AN and analyzed, respectively, on lanes 6−9 of a 6% polyacrylamide gel containing 7 M urea. The DNase I cleavage pattern of naked NX1P7 DNA is shown as reference (lanes 3−5). Locations of DNase I cleavage sites were determined by comparison to dideoxy A and C sequencing reactions (lanes 1 and 2).

2B, lane 6) is consistent with the positioning of the nucleosome in the NX1 sequence. At a higher histone to DNA weight ratios (see Figure 2A), bands (N2 and N3) with lower mobility on nondenaturing gels are observed—even aggregated nucleosomes (AN) that cannot enter the gel are produced—but the additional histones bound to the template caused no detectable changes in the DNase I footprints (Figure 2B, lanes 7−9). This indicates that only the NX1 DNA is wrapped around a histone octamer and gives rise to a normal nucleosome in the NX1P7 template. The additional histones bound to the template cause the observed decrease in the electrophoretic mobility of the template, but they do not have enough space to organize a second nucleosome and do not induce the nuclease cleavage pattern corresponding to folded nucleosomal DNA.

Transcription Near Physiological Ionic Strength. The transcription start site of the NX1P7 DNA template is located 18 bp from the end that does not contain the nucleosome positioning sequence (runoff transcript 221 nucleotides). Since the structure and dynamic properties of the nucleosome are dependent on the ionic strength conditions (Yager & van Holde, 1984; Daban & Cantor, 1989), it is necessary to try to approach the cellular conditions in transcription studies of chromatin. Unfortunately, the presence of 0.15 M NaCl inhibits T7 RNA polymerase almost completely (Chamberlin & Ring, 1973). However, we have obtained a high rate of RNA synthesis using NaCl concentrations as high as 50 mM, when the transcription buffer contains in addition 1.7−8 mM $MgCl_2$, 0.5−1 mM spermidine, 24 mM DTT, and 40 mM Tris-HCl (pH 8.0). Conductivity measurements (performed using NaCl solutions of different concentrations as reference) indicate that the ionic strength of this buffer is equivalent to 85 mM NaCl.

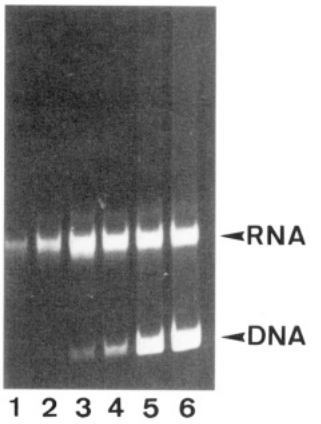

FIGURE 3: High transcription yields with small amounts of DNA template. The amounts of naked NX1P7 DNA used as template were (lanes 1−6) 0.005, 0.01, 0.05, 0.1, 0.5, and 1 μg, respectively.

This relatively high ionic strength poses additional problems that must be considered in order to carry out a reliable study of chromatin transcription. Taking into account that salt favors dissociation of nucleosomes (Yager & van Holde, 1984; Aragay et al., 1991), even if the concentration of the chromatin template is relatively high (we have used 63 μg of DNA/mL in chromatin samples), some degree of dissociation has to be expected when the ionic strength is equivalent to 85 mM NaCl. Note (see Figure 3) that small amounts of free DNA (i.e., about 1% of the total NX1P7 DNA used in our transcription experiments) can produce a high amount of RNA using the transcription conditions of this study. Thus, transcription of the chromatin template cannot be studied directly by monitoring the amount of synthesized RNA because it is not possible to know if this RNA is produced by transcription of the chromatin template or free DNA.

Transcription Causes Nucleosome Dissociation and Histone Redistribution within the Template. In previous reports (Aragay et al., 1988, 1991) we have shown that nondenaturing polyacrylamide gels containing TBE are very useful for the study of the association of histones with DNA under a wide variety of ionic strength conditions. As can be seen in Figure 4A, nondenaturing 6% polyacrylamide gels allow the direct analysis of the transcript and the NX1P7 chromatin template before and after transcription.

We have observed that only very high values of the core histone to DNA weight ratio inhibit the transcription reaction. At very low histone to DNA weight ratio, apparently there is no change in the intensity of the band corresponding to the positioned nucleosome core (band N1) at the end of the transcription (Figure 4A, lanes 4−6). At higher weight ratios (lanes 7−12), transcription is associated with a decrease in

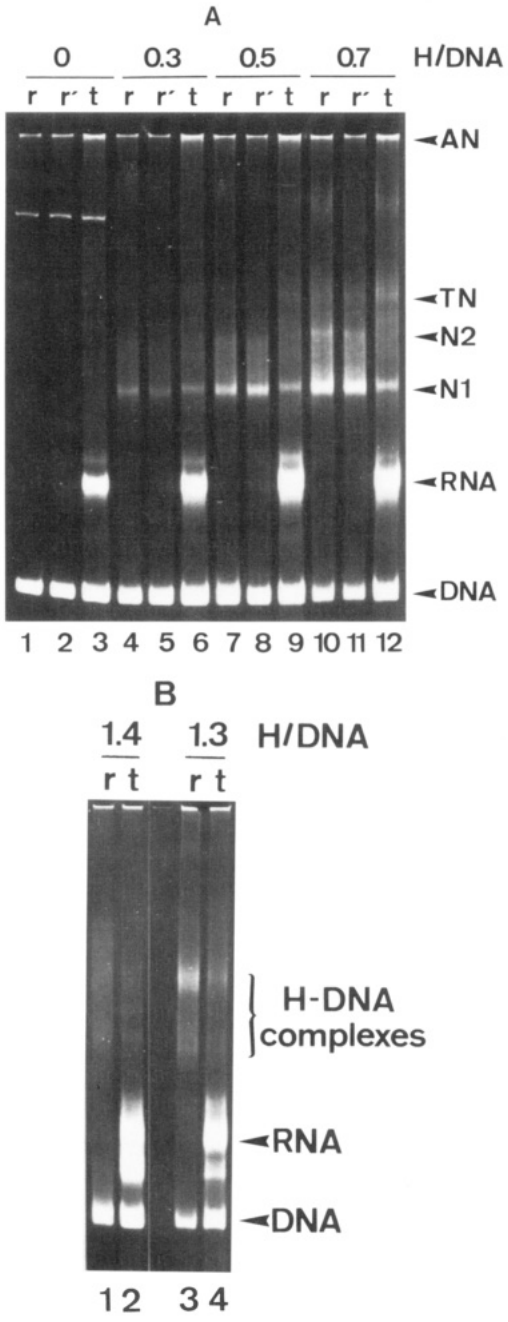

FIGURE 4: (A) Transcription of the short chromatin template containing the four core histones. (B) Transcription of NX1P7 DNA−(H2A,H2B) (lanes 1 and 2) and NX1P7 DNA−(H3,H4) (lanes 3 and 4) complexes. The input weight ratios of core histones to NX1P7 DNA are indicated at the top; t indicates samples transcribed for 30 min at 37 °C under standard conditions; r and r′ correspond to reference samples incubated for 30 min at room temperature and at 37 °C, respectively, but without T7 RNA polymerase and nucleotides.

the intensity of bands N1 and N2 and the appearance of a new band (TN). The formation of this band during transcription suggests that the passage of the RNA polymerase can induce histone redistribution within the template. Recently, Studitsky et al. (1994) and O'Donohue et al. (1994) have observed intramolecular histone rearrangements during transcription of short chromatin templates.

In addition, the results presented in Figure 4A (compare lanes 11 and 12) show that the decrease in the intensity of the N1 and N2 bands produced by transcription of complexes prepared at a histone to DNA weight ratio of 0.7 is accompanied by an increase in the intensity of the band corresponding to free NX1P7 DNA. This dissociation of NX1P7 DNA is much more clearly seen in samples with higher values of the histone to DNA weight ratio (see the results presented below; Figure 8, lanes 1 and 2 of panels B and C). As found for chromatin templates containing the four core histones, the results in Figure 4B indicate that the intensity of the ill-defined bands corresponding to NX1P7 DNA−(H2A,H2B) and NX1P7 DNA−(H3,H4) complexes decreases during transcription. In general, it is observed (see Table 1) that the amount of free DNA released in the transcription of the different chromatin templates used in this study increases with the input weight ratio of histones to DNA.

Table 1: Release of Free DNA Induced by Transcription of the Short Chromatin Template[a]

template		free DNA (%)	
histones	histone/DNA weight ratio	before transcription	after transcription
	0	100	100
core	1.1	62	95
	1.4	24	87
H2A,H2B	1.1	78	92
	1.4	73	96
H3,H4	1.1	55	59
	1.13	49	77

[a] Transcription conditions are the same as those of Figure 4.

Taken together, the results presented in this section indicate that during transcription histones can be redistributed within the DNA template and dissociated from the template, producing the observed accumulation of free NX1P7 DNA. In this paper we have focused attention on the study of the role of different elements that, in principle, could be related with nucleosome dissociation induced by transcription.

Transcription in the Presence of High Molecular Weight DNA Increases the Level of Dissociation of the Chromatin Template. As can be seen in Figure 5, when transcription is carried out in the presence of high molecular weight DNA, the decrease in the intensity of the chromatin template bands is higher than that observed in the absence of excess free DNA. This suggests that histones are released from the template and form complexes with the high molecular weight DNA.

Note that this decrease in the intensity of the N1 band is observed when the fraction of NX1P7 DNA that is not complexed with histones is very high (see Figure 5, lanes 9−12). This result suggests that the T7 RNA polymerase has no preference for the transcription of the naked DNA template and transcribes chromatin even in the presence of a large amount of naked template. Thus, in the absence of high molecular weight DNA, when the fraction of free NX1P7 DNA is really very high (as in the experiment shown in Figure 4A, lanes 4−6), it is also very likely that the chromatin template is transcribed. In this case there is no apparent change in the intensity of the N1 band because the released histones are associated with the free NX1P7 DNA fragments present at a relatively high concentration in the solution. Core histones are probably bound to the nucleosome positioning sequence of the acceptor NX1P7 DNA fragments and form a band having exactly the same mobility as the initial complex.

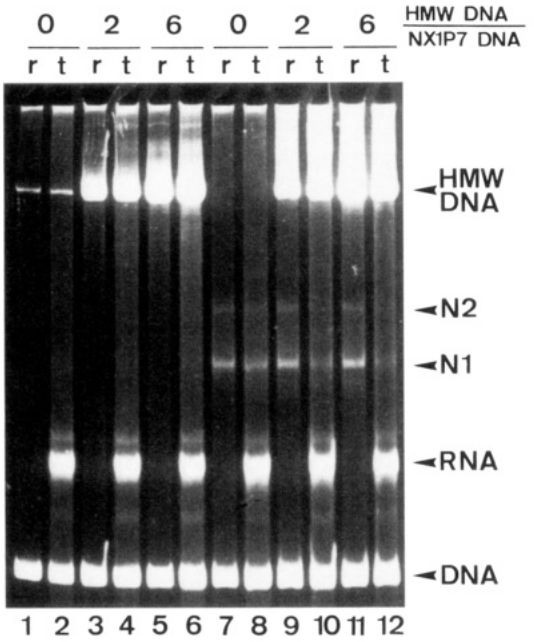

FIGURE 5: Transcription of the short chromatin template in the presence of free DNA of high molecular weight (HMW). The input weight ratios of core histones to NX1P7 DNA was 0 (lanes 1−6) and 0.4 (lanes 7−12). The HMW DNA to NX1P7 DNA weight ratios are indicated at the top; t indicates samples transcribed for 30 min at 37 °C under standard conditions; r correspond to reference samples incubated for 30 min at 37 °C, in the presence of T7 RNA polymerase but without GTP.

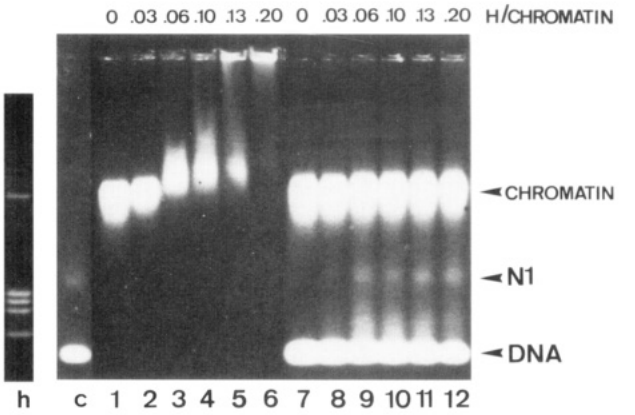

FIGURE 6: Association of core histones to native chromatin containing histones H1−H5 (lanes 1−6) and further transfer of excess histones to free NX1P7 DNA (lanes 7−12). The weight ratios of excess core histones to DNA of native chromatin are indicated at the top. The weight ratio of NX1P7 DNA to the DNA of native chromatin in lanes 7−12 was 0.4. Samples were analyzed on nondenaturing agarose gels containing 1.7 mM $MgCl_2$ and 90 mM Tris−borate (pH 8.3); in these gels the intensity of staining of nucleosome bands with ethidium bromide is very low in comparison to naked DNA (S. Bartolomé, A. Bermúdez, and J.-R. Daban, unpublished results). A sample without native chromatin (histone to NX1P7 DNA weight ratio = 0.5) is shown as reference (lane c). Histone composition of the native chromatin is shown in lane h; bands stained with Nile red (Daban et al., 1991) correspond, from top to bottom, to histones H1 (two faint bands), H5, H3, H2B, H2A, and H4.

On the other hand, in the absence of high molecular weight DNA, when the initial amount of free NX1P7 is very low (i.e., in samples with a high histone to DNA weight ratio; see the preceding section, Table 1, and Figure 8B,C), the histones dissociated during transcription cannot associate with free DNA. Under these conditions it seems reasonable to suggest that histones released from the transcribed templates can associate as excess histones with the nucleosomes assembled on other template DNA molecules, giving rise to aggregated complexes that cannot be resolved by the nondenaturing gel. This possibility, which in principle is compatible with previous studies on the association of excess histones with nucleosomes (Eisenberg & Felsenfeld, 1981; Stein et al., 1985; Aragay et al., 1991), is examined in the next section.

Core Histones Released by Transcription Can Be Bound as Excess Histones to Chromatin. The results presented above suggest that histones released during transcription could be associated with nucleosomes previously assembled on the NX1P7 DNA. In agreement with this interpretation, we have found that the addition of a 2-fold weight excess of chromatin depleted of histones H1−H5 (see Materials and Methods) to the transcription reaction of the short chromatin template results in a 4-fold increase in the amount of free NX1P7 DNA accumulated after transcription. These observations are consistent with previous findings showing that excess histones can be bound to nucleosome core particles (Eisenberg & Felsenfeld, 1981; Stein et al., 1985). In particular, in a previous electrophoretic study (Aragay et al., 1991), it has been found that the addition of excess histones to nucleosome cores or oligonucleosome core particles produces complexes that remain unresolved at the top of the gel.

However, all these previous studies have been performed using nucleosome core particles or chromatin without histones H1−H5. Therefore, it is of interest to know whether excess histones can also be bound to native chromatin containing the four core histones and histones H1−H5. In order to test this possibility, we have used agarose gels to analyze the interaction of core histones with native chromatin of high molecular weight. In agarose gels containing 1.7 mM $MgCl_2$, chromatin fragments are folded as the typical 30-nm fibers (Bartolomé et al., 1994) and form defined bands (S. Bartolomé, A. Bermúdez, and J.-R. Daban, unpublished results). As can be seen in Figure 6, in these gels fragments of chromatin containing ~30 nucleosomes form a band (lane 1) that becomes broad and disappears progressively when increasing amounts of core histones are added to this chromatin sample (lanes 2−6). This result demonstrates that chromatin containing core histones and histones H1−H5 (see lane h) can associate excess histones.

Further addition of free NX1P7 DNA to the resulting samples containing native chromatin complexed with excess histones results in the dissociated of these complexes (Figure 6, lanes 8−12). Excess core histones are transferred to NX1P7 DNA and form the normal N1 complex. Note that the band corresponding to native chromatin is recovered. Since this defined chromatin band is not observed when chromatin samples does not contain the normal amount of histones H1−H5 (S. Bartolomé, A. Bermúdez, and J.-R. Daban, unpublished results), these results indicate that histones H1−H5 remain bound to native chromatin.

Transcription of the short chromatin template in the presence of a large excess of native chromatin containing histones H1−H5 also gives rise to the accumulation of a significant amount of free NX1P7 DNA (Table 2). These results, and the observations considered above, suggest that core histones dissociated from NX1P7 DNA during tran-

Table 2: Transcription of the Short NX1P7 Chromatin Template in the Presence of Native Chromatin Containing Histones H1−H5

weight ratios		free NX1P7 DNA (%)	
core histone to NX1P7 DNA	DNA of chromatin to NX1P7 DNA	before transcription	after transcription
0	0	100	100
	4	100	100
0.8	0	62	75
	2	54	73
	4	43	74

scription can be bound to native chromatin. Presumably, at least in part, excess core histones bound to native chromatin are transferred to free DNA template and form reassembled nucleosomes. The fraction of excess histones irreversibly associated with the short chromatin template or chromatin cannot re-form nucleosomes and is responsible for the accumulation of free DNA template found at the end of the transcription reaction.

The High Affinity of Histones for ssDNA Suggests That Nucleosome Dissociation Is Not Due to the Formation of ssDNA by RNA Polymerase. A possible mechanism leading to nucleosome dissociation could be the destabilization of this structure produced by ssDNA formation during transcription. Thus, we have investigated the relative affinity of core histones for ssDNA and dsDNA. In an early study, Palter et al. (1979) showed that a histone octamer and two segments of ssDNA (140−160 nucleotides each) can form nucleosomelike particles. We have used a previously described method (Fernandez-Busquets & Daban, 1992) to purify the (+) and (−) strands of the NX1 nucleosome positioning sequence. In the presence of core histones these ssDNA samples become protected from digestion by micrococcal nuclease (Figure 7A), indicating that histones can associate with these particular ssDNA fragments. The titration experiments presented in Figure 7B show that the addition of increasing amounts of core histones to these samples results in the progressive disappearance of the electrophoretic bands corresponding to the different DNA forms. The amount of core histones required to produce the complete disappearance of the free DNA band is the same for both the (+) strand and dsDNA. In the case of the (−) strand a larger amount of histones is needed to produce an equivalent effect. Similar results have been obtained when the histone pairs H2A,H2B and H3,H4 have been used separately (data not shown).

We estimate that the apparent binding constant corresponding to the association of core histone octamers with the (+) strand of the NX1 fragment is about 5-fold higher than that corresponding to the association of histones with the (−) strand. Note, however, that this represents a difference in the free energy of binding of roughly 1 kcal/mol, which is only a small fraction of the total free energy decrease in nucleosome formation [about −10 kcal/mol, as estimated from the above results and from the dissociation constant in 0.2 M NaCl determined in previous studies (Aragay et al., 1991)]. Furthermore, the results obtained in competitive association experiments using ds and ssDNA from bacteriophage M13 (data not shown) indicate that in general the affinity of histones for ssDNA is similar to that found for dsDNA. All these findings allow us to conclude that it is very unlikely that the short ssDNA segments

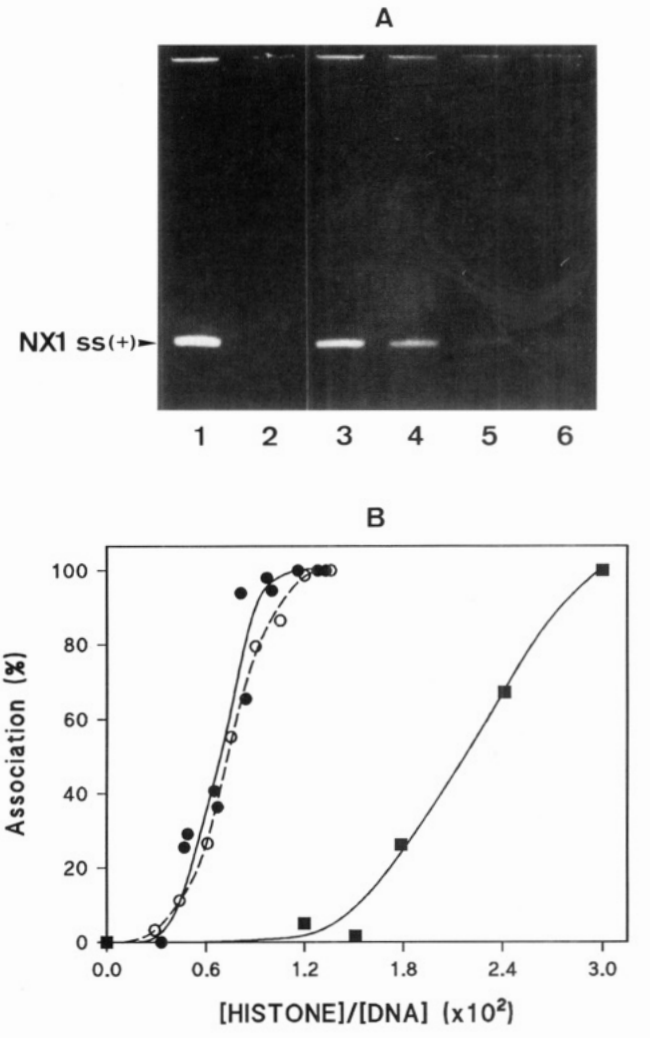

FIGURE 7: Association of the (+) and (−) strands of the NX1 fragment with core histones. (A) The (+) strand of the NX1 fragment complexed with core histones in 0.2 M NaCl was digested with a fixed amount of micrococcal nuclease for (lanes 3−6) 0, 2, 5, and 13 min, respectively. The free (+) strand (lane 1) was digested for 2 min (lane 2) under the same conditions. (B) Titration of the (+) (●) and (−) (■) strands of the NX1 fragment and of the native NX1 dsDNA (○, dotted line); the [histone]/[DNA] values correspond to the input ratio of core histone octamers to bp (dsDNA) or nucleotides (ssDNA).

produced transiently during transcription could be responsible for the nucleosome dissociation observed in the experiments shown above.

Histones Are Not Associated with the Synthesized RNA. Under the transcription conditions used in the present study, the final weight ratio of the synthesized RNA to the DNA template is approximately 7. Since the results obtained in titration and competitive association experiments (data not shown) under the ionic strength conditions of transcription indicate that RNA has a high affinity for core histones and can compete for histones in the presence of DNA, we have examined whether the synthesized RNA is involved in the nucleosome dissociation induced by transcription.

As can be seen in Figure 8, the complete digestion of the RNA accumulated at the end of transcription does not change the intensity of the bands of the chromatin template and free DNA (compare lanes 3 and 4 of panel A and lanes 2 and 3 of panels B and C). Thus, we conclude that the synthesized RNA is not associated with histones; otherwise the histones released after RNA digestion should have produced a

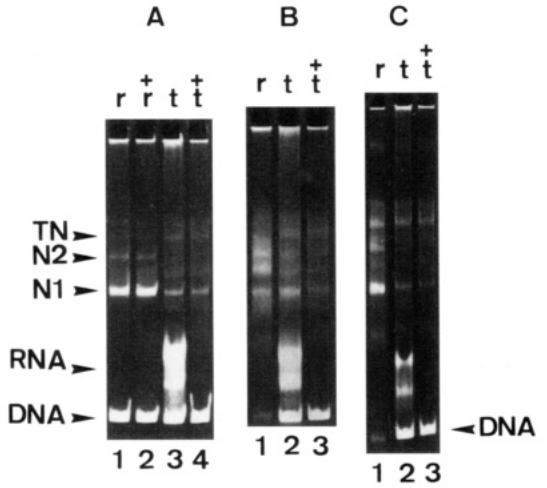

FIGURE 8: Digestion of RNA after transcription. The core histone to NX1P7 DNA weight ratios were 0.7 (A), 1.4 (B), and 1.6 (C). The transcribed (t) and reference samples (r, untranscribed) were extensively digested with RNase (lanes with +) and analyzed on nondenaturing polyacrylamide gels. Note that, at the high histone to DNA weight ratio used in panel C, band TN is observed before transcription.

remarkable decrease in the intensity of the free DNA band. We have also observed that the addition of RNA before the initiation of transcription has no effect on the stability of the chromatin template (data not shown). Although the possibility exists that the nascent RNA chains interact transiently with histones during the transcription reaction, all these observations indicate that the synthesized RNA is not involved in the nucleosome dissociation detected in the electrophoretic gels after chromatin transcription.

DISCUSSION

From the results presented above, we suggest a dynamic model for chromatin transcription in which histones released by RNA polymerase are transiently associated with chromatin and finally are reassembled with DNA to reform nucleosomes after the passage of the polymerase. The association of excess core histones and histones H2A,H2B and H3,H4 with nucleosome cores without histones H1−H5 has been previously demonstrated in various physicochemical studies (Eisenberg & Felsenfeld, 1981; Stein et al., 1985; Aragay et al., 1991), and in this work we have shown that folded chromatin containing core histones and histones H1−H5 can also bind excess histones. The high concentration of chromatin within the nucleus (Goodsell, 1993) favors the possibility that in vivo chromatin regions near transcribed DNA can associate excess histones. Furthermore, excess histones bound to nucleosome core particles (Stein et al., 1985; Aragay et al., 1991) or native chromatin containing H1−H5 (this work) can be transferred spontaneously to free DNA. However, the in vitro reactions of binding of excess histones produce aggregated structures that do not allow the complete transfer of these histones to DNA. This partial irreversibility explains the accumulation of free DNA template in the transcription reactions performed in this study. Nevertheless, it can be speculated that if within the nucleus these reactions occur in the localized regions where transcription is taking place, the attachment of chromatin to the nuclear matrix (Jack & Eggert, 1992) may prevent the formation of irreversible aggregates and histones released during transcription could be rapidly associated with chromatin and transferred to uncomplexed DNA following a completely reversible mechanism.

In addition to the nucleosome dissociation reaction considered above, Studitsky et al. (1994) and O'Donohue et al. (1994) have observed that transcription induces nucleosome translocation within the template. In agreement with these authors, we have found that the presence of excess free DNA in the transcription reaction favors nucleosome dissociation. Our results indicate that the presence of chromatin also facilitates the dissociation mechanism. In addition, the relatively high ionic strength present in our transcription reactions has reduced the activation energy required for nucleosome dissociation and, presumably, has also facilitated the mechanism involving histone displacement from the template.

Taking into account the tripartite organization of the core histone octamer (Arents & Moudrianakis, 1993) and the linear arrangement of histones H2A,H2B−$(H3,H4)_2$−H2A,-H2B along 146 bp of DNA in the nucleosome (Shick et al., 1980), it seems reasonable to suggest that the transfer of histones to DNA or chromatin during transcription of a nucleosome could take place progressively by the successive release of histone pairs. Our results indicate that T7 RNA polymerase can transcribe templates without H2A,H2B or H3,H4. Alternatively, if, as suggested by O'Neill et al. (1993) from studies with cross-linked histones, octamers are released as intact units from DNA by the polymerase, it is very likely that they will become rapidly dissociated into H2A,H2B dimers and H3,H4 tetramers since the lifetime of octamers is very short at physiological ionic strength (Feng et al., 1993). The spontaneous reactions of transfer and exchange of H2A,H2B and H3,H4 involved in the in vitro formation of core particles (Aragay et al., 1988, 1991; Samsó & Daban, 1993) could participate in the mechanism of nucleosome reassembly after transcription.

Our results exclude the possibility that the RNA polymerase induces nucleosome dissociation due to the formation of segments of ssDNA during transcription. Moreover, our results show that the dissociated histones are not bound to the synthesized RNA. Thus, the question of how RNA polymerase dissociates nucleosomes remains open. The possibility that the polymerase generates topological stress capable of nucleosome dissociation (Liu & Wang, 1987; Pfaffle & Jackson, 1990; Lee & Garrard, 1991; Clark & Felsenfeld, 1992) is difficult to consider with the noncircular short chromatin template used in this study. A second possibility is that the relatively large amount of chemical energy involved in the transcript elongation reactions (Erie et al., 1992) provides enough mechanical energy for the movement of the polymerase along the template and for the direct displacement of the histones bound to DNA. In fact, since the free energy change for RNA synthesis is about −2 kcal/mol of nucleotide polymerized in each single-nucleotide elongation step (Erie et al., 1992), the decrease of free energy after the addition of few nucleotides to the transcript in the process of elongation is higher than the total amount of energy stabilizing the core particle (about −10 kcal/mol, see above). Considering that the 3′ region of the nascent RNA transcript is base-paired to the template strand, the elongation of the RNA chain generates a force that can be exerted by the polymerase on the transcribed nucleosome and can induce its dissociation. This transcript elongation force has also been detected by Fujita and Silver (1993) in another system.

These authors have found that in the process of transcription T7 and T3 RNA polymerases induce the dissociation of streptavidin−biotinylated DNA complexes, which have a very strong binding constant (on the order of 10^{12} M^{-1} in this system).

ACKNOWLEDGMENT

We thank the staff of the Servei de Seqüenciació de DNA (CID-CSIC) for their assistance. We are grateful to Miquel Cabañas and Irene Martínez for their help with the HPLC equipment and to Dr. Jaume Farrés for a useful comment.

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BI942885V