The binding of a Fos/Jun heterodimer can completely disrupt the structure of a nucleosome

Kean Wooi Ng¹, Patricia Ridgway¹,
Donna Ruth Cohen² and
David John Tremethick^{1,3}

Divisions of ¹Biochemistry and Molecular Biology and
²Immunology and Cell Biology, The John Curtin School of Medical
Research, The Australian National University, PO Box 334,
Canberra ACT 2601, Australia

³Corresponding author
e-mail: david.tremethick@anu.edu.au

An important first step in the chromatin remodelling process is the initial binding of a transcriptional activator to a nucleosomal template. We have investigated the ability of Fos/Jun (a transcriptional activator involved in the signal transduction pathway) to interact with its cognate binding site located in the promoter region of the mouse *fos*-related antigen-2 (*fra-2*) promoter, when this site was reconstituted into a nucleosome. Two different nucleosome assembly systems were employed to assemble principally non-acetylated or acetylated nucleosomes. The ability of Fos/Jun to interact with an acetylated or an unacetylated nucleosome differed markedly. Fos/Jun bound to an unacetylated nucleosome with only a 4- to 5-fold reduction in DNA binding affinity compared with naked DNA. Strikingly, the binding of Fos/Jun to a single high-affinity site incorporated into an acetylated nucleosome resulted in the complete disruption of nucleosomal structure without histone displacement. Moreover, this disruption was sufficient to facilitate the subsequent binding of a second transcription factor.

Keywords: chromatin/histone acetylation/nucleosome disruption/transcription factors

Introduction

The establishment of an active transcription complex on a eukaryotic RNA polymerase II promoter in chromatin is an extremely complex process involving an interplay between many different components, including specific DNA sequences (and structures), histones, non-histone chromosomal proteins, transcriptional activators (and repressors), architectural proteins and the basal transcription machinery (Workman and Buchman, 1993; Becker, 1994; Wallrath *et al.*, 1994; Wolffe, 1994). Thus, it is not surprising that the promoter of an active gene is assembled into a highly organized and complex structure, although the precise molecular steps by which such a structure is assembled is far from being understood. Clearly, the reproduction of an active from an inactive chromatin structure *in vitro* will be crucial for unravelling these molecular steps. In addition, to understand the complex

nature of this process, it will be necessary to disassemble the activation process into amenable areas of research.

A considerable body of evidence, from both *in vivo* and *in vitro* studies, has shown that the assembly of a eukaryotic promoter into chromatin can block the establishment of a transcription complex (Workman and Buchman, 1993; Becker, 1994; Wallrath *et al.*, 1994; Wolffe, 1994). This repression of transcription can occur at two distinct structural levels. Incorporation of an important promoter element into a nucleosome can sterically prevent the stable binding of a transcription factor to its recognition sequence (Tremethick *et al.*, 1990; Taylor *et al.*, 1991; Workman *et al.*, 1991). Transcription factor access can be further restricted by the subsequent folding of an array of nucleosomes into more compact higher-order structures (Kamakaka *et al.*, 1993; Bouvet *et al.*, 1994). These results have led to the proposal that nucleosome repression of transcription is one important mechanism by which a large number of genes in the eukaryotic nucleus remain repressed despite the presence of ubiquitous transcription factors (Workman and Buchman, 1993). For a gene to be activated, the nucleosomal fibre must first be de-condensed and then nucleosomes must be removed from important promoter elements. This two-step change in the chromatin structure, associated with the transcriptional activation process, is reflected by a differential increase in the accessibility of an active gene towards DNase I, i.e. the chromatin domain of an active gene, and key regulatory sequences become sensitive and hypersensitive to digestion by DNase I, respectively (Wallrath *et al.*, 1994).

An important first step in understanding the extremely complex process of active transcription complex formation within a chromatin environment will be to understand how positive activators interact with a single nucleosome. Such an approach, involving the reconstitution of a nucleosome encompassing a transcription factor binding site, has generated much information concerning transcription factor–nucleosome interactions. For example, different transcription factors display a wide range of binding affinities with regard to their ability to interact with a defined sequence within a nucleosome (Taylor *et al.*, 1991; Li *et al.*, 1994; Wechsler *et al.*, 1994; Blomquist *et al.*, 1996). Also, the ability to interact with a nucleosome can depend on the rotational position of a transcription factor binding site, i.e. whether the site faces towards or faces away from the histone octamer (Archer *et al.*, 1991; Li and Wrangé, 1995). The location of a binding site relative to the centre of a nucleosome is also important, as DNA sequences located at the end of a nucleosome tend to be more accessible than those located towards the centre of the nucleosome where histone–DNA contacts are the strongest (Taylor *et al.*, 1991; Vettese Dadey *et al.*, 1994; Adams and Workman, 1995). This is best illustrated by the interaction of GAL-4 with nucleosome cores containing five GAL-4 binding sites (Vettese Dadey

et al., 1994). In contrast to naked DNA, GAL-4 binding to the nucleosome occurred in a cooperative manner, with initial binding at the site at the end of the nucleosome core, which in turn facilitated binding to more internal sites. In these studies, transcription factor binding alone was not sufficient to cause nucleosome displacement, which was achieved when nucleosome assembly factors such as nucleoplasmin were present in the binding reaction (Chen *et al.*, 1994; Walter *et al.*, 1995). These assembly factors facilitate nucleosome displacement by removing histones from ternary complexes. Nucleosome displacement was not observed when only a single GAL-4 site was incorporated into a nucleosome.

Despite the significance of this work, only a limited number of transcription factors have been used in similar investigations. Moreover, in many cases, artificial promoters and transcription factors have been employed. Therefore, it is necessary to undertake a more extensive study employing natural promoters with a greater variety of transcription factors that possess different DNA binding and activation domains (Workman and Buchman, 1993). In this investigation, we chose to examine the ability of AP-1 (Fos/Jun) to bind to a nucleosome. AP-1 is a ubiquitous transcription factor involved in signal transduction pathways.

Extracellular signals regulate the activity of many different kinds of transcription factors. A variety of these signals stimulate the activity of the transcription factor AP-1 (Karin, 1995). Following activation of AP-1 activity, gene expression is re-programmed by the transcriptional activation (or repression) of AP-1 target genes (Angel and Karin, 1991). AP-1 consists of a mixture of polypeptides that are encoded by the immediate-early genes of the *fos* (*c-fos*, *fosB*, *fra-1* and *fra-2*) and *jun* (*c-jun*, *junB* and *junD*) families. Both Fos and Jun family members share a well-conserved region containing the basic DNA-binding domain and leucine zipper dimerization motif (bZip) (Angel and Karin, 1991; Kerppola and Curran, 1991a). The bZip domains of Fos and Jun exist as continuous α -helices which pair to form a coiled-coil through their leucine zippers. The two helices diverge at the basic region to form a Y-shaped structure. Each basic arm of the Y structure interacts with the major groove of the DNA in a scissors grip manner (Glover and Harrison, 1995). Both Fos and Jun contribute to the transactivation function of the AP-1 complex through binding to the AP1 consensus complex (TGA G/C TCA) (Angel and Karin, 1991; Kerppola and Curran, 1991a). Since AP-1 induces transcription by binding to enhancer and promoter elements of target genes, it would be expected that, at least in some cases, AP-1 would encounter its binding site incorporated into chromatin. Whether AP-1 can interact with a nucleosome is currently not known.

In this investigation, we present novel data indicating that Fos/Jun can disrupt the structure of a nucleosome. Moreover, multiple DNA binding sites for AP-1 are not required for this disruption process. These findings suggest that AP-1 may play an important role in the chromatin remodelling process.

Results

Nucleosome assembly

In this study, we have investigated the ability of Fos/Jun to interact with its binding site when this site is incorpor-

ated into a nucleosome. The model system we employed involves the assembly of a single nucleosome onto a 180 base pair (bp) DNA fragment. Two different nucleosome assembly systems were used. The first procedure is a well-characterized and widely used method involving the transfer, under high-salt conditions, of a nucleosome from histone H1-depleted donor chromatin (isolated from chicken red blood cells) to a ^{32}P end-labelled DNA fragment. Following dilution of the mixture from high-salt to physiological conditions, donor chromatin is removed by precipitation with MgCl_2 (see Materials and methods). The nucleosome assembled consists primarily of unacetylated histones (Dimitrov *et al.*, 1993). The second assembly system, which assembles nucleosomes easily and efficiently under physiological conditions, utilizes the N1/N2-(H3,H4) complex isolated from *Xenopus laevis* ovaries (Tremethick and Frommer, 1992). Histone H4 in the complex is present in the diacetylated form and histone H3 is similarly modified (Zucker and Worcel, 1990). To assemble a complete nucleosome containing an acetylated H3/H4 tetramer, as previously described (Tremethick *et al.*, 1990; Zucker and Worcel, 1990; Tremethick and Frommer, 1992; Tremethick and Drew, 1993; Tremethick, 1994; Tremethick and Hyman, 1996), the N1/N2-(H3,H4) complex is supplemented with chicken H2A/H2B. In the absence of histone H2A/H2B, histone H3/H4 containing subnucleosomal particles are assembled that can supercoil DNA and protect DNA from micrococcal nuclease digestion (Tremethick *et al.*, 1990; Zucker and Worcel, 1990). Importantly, using this *in vitro* nucleosome assembly system, authentic chromatin with different physiological nucleosome repeat lengths can be assembled when histone H1 and/or HMG proteins are added to the assembly reaction (Tremethick and Frommer, 1992; Tremethick and Drew, 1993; Tremethick and Hyman, 1996).

The 180 bp DNA template used for most of the experiments reported here encompasses the region from -65 to +129 of the mouse *fra-2* promoter (see Figure 8B) (Foletta *et al.*, 1994). This particular promoter was chosen because, in addition to an AP-1 consensus site (TGAC-TCA), two high-affinity binding sites for the HMG-box protein SRY (a consensus and non-consensus site, sites 1 and 2 respectively in Figure 8B) are also present on this DNA fragment (the ability of SRY to bind to a nucleosome is currently under investigation and is not the subject of this study). SRY is a central component involved in testis development (Berta *et al.*, 1990). There is *in vitro* evidence that binding of AP-1 and/or SRY to these sites is significant, as expression of Fos/Jun or SRY in transient transfection studies stimulates the expression of *fra-2* promoter reporter constructs containing these sites (V.C.Foletta and D.R.Cohen, unpublished data). The 180 bp fragment used in most of these experiments was generated such that the AP-1 binding site was located centrally in the fragment. The binding of AP-1 to a nucleosome reconstituted on this fragment or to naked DNA (mock-assembled) was monitored by gel mobility shift assays and DNase I footprinting.

First, it was necessary to establish that a complete nucleosome is generated following mixing of the N1/N2-(H3,H4) complex with histones H2A/H2B and template DNA. In Figure 1, a gel mobility shift assay was used to

Fig. 1. Both histone pairs H3/H4 and H2A/H2B are required to form a stable nucleosomal structure. Assembly reactions containing the N1/N2-(H3,H4) complex, histones H2A/H2B, 1 ng of a labelled 180 bp *fra-2* DNA fragment, and 100 ng of a 180 bp carrier DNA fragment were carried out as described in Materials and methods. Lane 1 and lanes 2–6 received 4.0 and 4.5 μ l of the N1/N2-(H3,H4) complex, respectively. Lane 7 did not receive any H3/H4 complex. Lanes 1 and 2 received no H2A/H2B. Lanes 3–7 received 2.5, 2.0, 1.5, 1.0 and 2.0 μ l of histones H2A/H2B, respectively.

demonstrate the formation of a distinct shifted complex when all of these assembly components were incubated together. Neither histones H2A/H2B nor histones H3/H4 alone incubated with template DNA produced a stable complex under the conditions employed here (Figure 1, compare lanes 3 and 7, and lanes 1 and 2, respectively). Only when all four histones were present was a stable complex observed, which implies that the reconstituted complex consists of all four histones. Results from other work has indicated that a histone H3/H4 tetramer is stable during the gel mobility shift assay (Lee *et al.*, 1993). We attribute this difference to the fact that histones H3/H4 in the N1/N2-(H3,H4) complex are modified, thereby weakening histone H3/H4–DNA interactions. Importantly, our result suggests that to achieve nucleosome displacement or disruption—as judged by the gel mobility shift assay—only histone H2A/H2B interactions within the nucleosome must be altered.

Other evidence demonstrating that this specific shifted histone complex is a nucleosome include (data not shown): (i) the mobility shift of the reconstituted histone complex is similar to that observed when a nucleosome is assembled onto the 180 bp fragment using the high-salt direct transfer method; (ii) the assembled histone product protects 145–150 bp of DNA from micrococcal nuclease digestion; (iii) analysis of the protein content of the shifted histone complex reveals the presence of all four histones; and (iv) DNase I digestion of the histone–DNA complex containing bent DNA sequences results in a 10–11 bp digestion ladder (see Figure 6B) which is identical to the ladder obtained when this DNA template is reconstituted into a nucleosome by the high-salt assembly procedure. Therefore, we have concluded that the stable shifted complex observed in Figure 1 is a nucleosome. Importantly, by carrying out careful histone titrations, at least 90% of the labelled DNA template can be assembled into a nucleosome, which is important when carrying out DNase I footprinting experiments as this removes the possibility that any footprint observed is due to un-assembled DNA.

The binding of a Fos/Jun heterodimer to nucleosomal DNA

Using the gel mobility shift assay, we investigated whether Fos/Jun can bind to a nucleosome. In Figure 2A, a nucleosome was assembled using the N1/N2-(H3,H4) complex plus pure histones H2A/H2B such that ~50% of the DNA template was reconstituted. Titration of Fos/Jun onto naked DNA (mock-reconstituted) produced a specific complex that migrated significantly more slowly than the DNA probe (Figure 2A, compare lanes 1 and 3). This complex would be equivalent to the binding of a single Fos/Jun heterodimer to the DNA probe. As observed for many transcription factors, addition of higher concentrations of Fos/Jun produced shifted complexes that migrated even more slowly than the initial specific complex formed, and is probably due to protein–protein interactions yielding oligomeric complexes (Figure 2A, compare lanes 3 and 9). When increasing amounts of Fos/Jun were added to the assembled nucleosome, the transcriptional activator first bound to the free probe (Figure 2A, lanes 4 and 6), indicating its preference for naked DNA. However, once the free DNA probe was almost gone, the Fos/Jun dimer bound to the nucleosome, as evidenced by the gradual loss of the complex corresponding to nucleosome alone. By 2.4 footprinting units (fpu) (only 2.4-fold more protein than that needed to produce a footprint on naked DNA), the nucleosome complex was completely gone. Surprisingly, no super-shifted complex, equivalent to an AP-1 molecule bound to a nucleosome, was obvious. The only complexes observed migrated in identical positions to that observed when AP-1 bound to naked DNA (Figure 2A, compare lanes 6, 8 and 10 with lanes 5, 7 and 9). This experiment has been reproduced numerous times with many different Fos/Jun and histone preparations.

A similar experiment was performed employing nucleosomes assembled using the high-salt transfer method (Figure 2B). Importantly, under identical binding conditions to those used in Figure 2A (see Materials and methods), Fos/Jun bound to the assembled nucleosome to produce a specific complex that migrated more slowly than a free DNA–Fos/Jun complex (Figure 2B, compare lane 10 with lanes 3 and 11). This demonstrates that Fos/Jun–nucleosome complexes can be resolved from Fos/Jun–free DNA complexes in the gel mobility shift assay, and suggests that the result shown in Figure 2A may be interpreted as Fos/Jun complexes disrupting or displacing nucleosomes. In addition, Figure 2B shows that Fos/Jun has a high affinity for an unacetylated nucleosome as only 4 fpu of Fos/Jun was required to produce a Fos/Jun–nucleosome complex. Footprinting experiments have revealed that 5 fpu of Fos/Jun was required to produce total protection of the AP-1 site on the nucleosome assembled from chicken chromatin (data not shown).

In Figure 2A, nucleosome assembly reactions were carried out in the presence of 3 mM ATP (the function of ATP in this reaction, distinct from its role in nucleosome spacing, is to chelate Mg^{2+} , which is an inhibitor of the nucleosome assembly reaction; Tremethick and Frommer, 1992). To investigate whether exogenous ATP is required for this apparent disruption of nucleosomal structure, an identical experiment to that described in Figure 2A was carried out, except that ATP was omitted from the assembly reaction and replaced with EDTA (Figure 2C). Figure 2C

Fig. 2. Fos/Jun can disrupt an acetylated nucleosome. (A) Mock-assembly reactions (lanes 1, 3, 5, 7 and 9) and acetylated nucleosomes, assembled using the N1/N2-(H3,H4) complex plus histones H2A/H2B and the 180 bp *fra-2* DNA fragment (containing a centrally located single AP-1 binding site) (lanes 2, 4, 6, 8 and 10), were incubated with increasing concentrations of Fos/Jun (in terms of fpu, as indicated) and then resolved on a mobility shift gel. Complexes representing the nucleosome and Fos/Jun bound to naked DNA are indicated. Uncomplexed DNA is shown as free DNA.

(B) Nucleosomes containing the 180 bp *fra-2* fragment were assembled using the high-salt transfer protocol employing histone H1-stripped chicken chromatin as described in Materials and methods. Mock-assembly reactions (lanes 1, 3, 5, 7, 9 and 11) and nucleosomes (lanes 2, 4, 6, 8 and 10) received increasing concentrations of Fos/Jun as shown. (C) Nucleosome assembly reactions were carried out in an identical manner to that described in (A) except that ATP was replaced with EDTA (see Materials and methods).

shows that when increasing amounts of Fos/Jun complexes were added to assembled nucleosomes, the only DNA-protein complexes observed migrated in identical positions to that of Fos/Jun-naked DNA complexes. This observation mimics the result of Figure 2A. Therefore, we can conclude that exogenous ATP is not required for this apparent ability of Fos/Jun to disrupt the structure of a nucleosome.

In these experiments, we used a DNA probe that was 180 bp in length because it has been shown that an octamer can organize at least 160 bp of DNA, with histones contacting DNA as far as 90 bp from the dyad (Pruss and Wolffe, 1993). Similarly, it has been demonstrated that the histone octamer can protect 168 bp of DNA from digestion by micrococcal nuclease (Weischet *et al.*, 1979). Clearly, histones can interact with DNA

outside the core particle. However, the strongest histone-DNA interactions occur within the core particle and in particular near the dyad. If an AP-1 complex is capable of disrupting the nucleosome by binding to its cognate site, presumably it must disorganize strong histone-DNA contacts in the core particle in order to dismantle the nucleosome. Therefore, we were interested to determine whether Fos/Jun would disrupt a nucleosome if its DNA binding site was located at the end of the 180 bp fragment where histone-DNA interactions are weak and not crucial for maintaining the integrity of the nucleosome. To address this issue, two additional DNA probes were synthesized by PCR such that the AP-1 binding site was positioned either midway between the centre and the 3' end of the fragment or 8 bp from the 3' end of the fragment. When the Fos/Jun binding site was located midway between the

Fig. 3. The location of the AP-1 binding site within the nucleosome determines whether the nucleosome is disrupted. Mock-assembly reactions (lanes 1, 3, 5, 7, 9, 11, 13, 15 and 17) and acetylated nucleosomes (lanes 2, 4, 6, 8, 10, 12, 14, 16 and 18) were assembled by incubating the N1/N2-(H3,H4) complex and histones H2A/H2B with three different 180 bp *fra-2* DNA fragments. Each DNA fragment contains a AP-1 binding site positioned at different locations (as shown). Following nucleosome assembly, Fos/Jun was added.

centre and the end of the probe, Fos/Jun complexes disrupted the structure of the nucleosome, to a similar extent as that observed when the site was located in the centre of the fragment (Figure 3, compare lanes 10 and 12 with lanes 4 and 6). In contrast, when the binding site was located at the end of the DNA fragment, a super-shifted complex was observed, corresponding to a Fos/Jun-nucleosome complex. Therefore, a Fos/Jun molecule and a histone octamer can coexist on the same template when the AP-1 binding site is located in a region where strong histone-DNA contacts do not exist. Thus, the location of the AP-1 binding site within the nucleosome determines whether the nucleosome is disrupted or remains intact. Further, this result demonstrates that the apparent disruption observed is absolutely dependent on DNA binding and rules out trivial explanations such as a contaminating protease associated with the Fos/Jun complex.

We next wished to determine whether nucleosome disruption by Fos/Jun complexes is a function of DNA binding, or if other domains in the AP-1 proteins are required to exert this effect. To investigate this, we used a heterodimeric complex consisting of truncated Fos (amino acids 116–211) (wbFos) and Jun (amino acids 224–334) (wbJun) polypeptides to determine if these proteins could disrupt the structure of a nucleosome (Figure 4). Jun (224–334) encompasses only the bZip region of the c-Jun protein, whereas Fos (116–211) contains the bZip region plus a short stretch of glutamic acid residues immediately N-terminal to the basic region shown to be a transcriptional activation domain in *in vitro* transcription assays (Abate *et al.*, 1990). In the gel mobility shift assay system employed here, the Fos (116–211)/Jun (224–334)-DNA complex migrated slightly faster than the nucleosome. Like the full-length dimeric complex, a complex consisting of these truncated proteins was able to disrupt an acetylated nucleosome, as evidenced by the

Fig. 4. The bZip regions of Fos and Jun are sufficient to disrupt the nucleosome. Acetylated nucleosomes (lanes 2, 4, 6 and 8) containing a 180 bp *fra-2* DNA fragment in which the AP-1 binding site is centrally located, and mock-assembly reactions (lanes 1, 3, 5 and 7) were assembled as described in the legend of Figure 2A and then incubated with increasing concentrations of Fos (116–211)/Jun (224–334) complex (wbFos/wbJun) (as indicated).

gradual replacement of the nucleosome complex with a complex corresponding to Fos (116–211)/Jun (224–334)-free DNA (Figure 4, compare lanes 2 and 8 with lane 7). Clearly then, the ability of Fos/Jun to disrupt a nucleosome may simply be the consequence of DNA binding, since the amino acid residues important for nucleosome disorganization include the residues critical for DNA binding, i.e. the bZip region. This result does not rule out the possibility that nucleosome disruption may depend on critical protein-protein interactions between histones and

Fig. 5. Fos/Jun can disrupt a nucleosome that contains *fra-1* promoter sequences. Acetylated nucleosomes incorporating either 180 bp of the *fra-1* promoter (containing two AP-1 binding sites) or 180 bp of the *fra-2* promoter (containing a single AP-1 binding site) were assembled using the N1/N2-(H3,H4) complex and histones H2A/H2B. Assembled nucleosomes (lanes 2, 4, 6, 8, 10, 12 and 14), or mock reactions (lanes 1, 3, 5, 7, 9, 11 and 13), were then incubated with increasing concentrations of Fos/Jun (as shown).

the Fos/Jun complex. However, if this is the case, the key amino acid residues of the activator complex important for this interaction lie in the bZip region of Fos and/or Jun, or the acidic region next to the bZip region of Fos. Future work, including cross-linking studies, will clarify this issue.

Can Fos/Jun disrupt the structure of a nucleosome if a different promoter region containing an AP-1 binding site is incorporated into an acetylated nucleosome? The rat *fra-1* promoter contains two AP-1-like binding sites near the start site of transcription, with the centre of the two sites separated by 57 bp (D.R.Cohen, unpublished data; see also Bergers *et al.*, 1995). Both of these AP-1 sites have a 4- to 5-fold lower affinity for Fos/Jun compared with the *fra-2* AP-1 site used earlier (data not shown). We carried out identical experiments to those described above, employing 180 bp DNA fragments that contain one or other of the *fra-1* AP-1 binding sites, or a fragment containing both of these sites. Essentially the same results were obtained with all three *fra-1* promoter fragments, namely that the addition of Fos/Jun was able to disrupt the structure of the nucleosome (Figure 5 and data not shown). An ~4-fold concentration of Fos/Jun was required to disrupt a nucleosome containing the *fra-1* AP-1 sites compared with a nucleosome containing the *fra-2* promoter site (Figure 5, compare lanes 8 and 14). These results demonstrate that the ability of Fos/Jun to disrupt the structure of a nucleosome is not promoter-specific. Importantly, this result also shows that the disruption of the nucleosome caused by Fos/Jun is dependent upon DNA binding and that the concentration of Fos/Jun required to disrupt the nucleosome depends on its affinity for the binding site.

The binding of a Fos/Jun complex to the *fra-2* promoter can completely disrupt the structure of a nucleosome

We wanted to use DNase I footprinting to confirm that Fos/Jun can indeed disrupt the structure of a nucleosome,

by exploiting the fact that a rotationally positioned nucleosome displays a different DNase I pattern compared with naked DNA. The digestion pattern of a positioned nucleosome consists of hypersensitive cleavage sites spaced at ~10 bp intervals (Archer *et al.*, 1991; Li and Wrangé, 1995). If such a digestion pattern is observed after assembling an acetylated nucleosome onto the DNA probe, the disruption of the nucleosome by Fos/Jun would result in the loss of the 10 bp ladder. However, digestion of reconstituted acetylated nucleosomes did not yield a 10 bp digestion ladder, indicating that the nucleosome assembled onto the DNA probe was not rotationally positioned. Similarly, no such ladder was observed when an unacetylated nucleosome was assembled onto the fragment using the high-salt transfer method (data not shown).

Therefore, we inserted a DNA bending sequence (two tandem 20 bp repeats of the GT sequence known to induce rotational phasing) into the 180 bp *fra-2* promoter region fragment 3' of the AP-1 site, even though this alters the natural context of the promoter region. This bending sequence has been used previously to direct a nucleosome next to a c-Myc/Max binding site (Wechsler *et al.*, 1994). Gel mobility shift assays employing this DNA fragment assembled into an acetylated nucleosome revealed that ~3- to 4-fold more Fos/Jun was required to disrupt the nucleosome compared with when the unmodified *fra-2* fragment was used (not shown). Figure 6, which analyses the binding of Fos/Jun binding to a rotationally positioned nucleosome by DNase I footprinting, confirms this result.

The binding of Fos/Jun complex (1 fpu) to naked DNA produced a complete footprint that encompassed the AP-1 consensus sequence (Figure 6, compare lane 3 with lane 1). Partial protection of the DNA further 3' of the consensus site was also observed (see Figure 8B for a summary of the footprinting data). Importantly, this figure clearly shows that Fos/Jun can indeed disrupt the structure of a nucleosome. Compared with the DNase I digestion profile of naked DNA (Figure 6, lanes 1 and 7), the

Fig. 6. The binding of Fos/Jun to rotationally positioned acetylated nucleosomes. The binding of Fos/Jun to acetylated nucleosomes containing a modified 180 bp *fra-2* DNA fragment, in which a DNA bending sequence was inserted 3' of the AP-1 binding site, was analysed by DNase I footprinting. Mock-assembly reactions and reconstituted nucleosomes were incubated with increasing concentrations of Fos/Jun as indicated. Arrowheads show sites of enhanced cleavage in nucleosomal DNA separated by areas of increased protection. ● indicates the appearance of naked DNA-specific DNase I cuts.

digestion profile of the reconstituted nucleosome clearly demonstrated a moderate 10 bp digestion ladder (Figure 6, lanes 8 and 18) (a very strong 10 bp ladder was not observed probably due to the fact that only two tandem GT bending sequences were utilized, that these bending sequences were located towards the end of the DNA fragment which is not the optimal position for these bending elements, and that—in contrast to other similar studies which use DNA fragments that are only ~150 bp in length—our DNA probe was significantly longer). When a Fos/Jun titration was performed, strikingly, the 10 bp ladder gradually disappeared and was replaced by the digestion pattern of naked DNA. This loss of the 10 bp ladder corresponded to the occupancy of the AP-1 site; that is, at the point at which 8 fpu of Fos/Jun was present

in the reaction, when 80% of the AP-1 sites are occupied, the DNase I digestion profile was essentially the same as naked DNA.

Unexpectedly, at the higher Fos/Jun concentrations (8 fpu) required to eliminate the 10 bp DNase I ladder, a second low-affinity site for Fos/Jun is observed upstream of the AP-1 consensus site (Figure 6, see vertical bar next to lanes 6 and 13; also see Figure 8B for a summary). Binding to this site occurs at 8 fpu, irrespective of whether a nucleosome is assembled onto the DNA probe. Nevertheless, we can not exclude the possibility that the binding of two Fos/Jun heterodimers is required for nucleosome disruption.

Rather than Fos/Jun binding causing disruption of nucleosomal structure, another possible explanation for

the loss of the 10 bp ladder is that Fos/Jun may induce nucleosome sliding. We do not believe this is the case because, as demonstrated in Figure 3, such a triple complex comprising DNA, a histone octamer, and an AP-1 complex would yield a super-shifted complex in a mobility gel shift assay. This is clearly not observed. Moreover, studies on nucleosome mobility have shown that different translational positions of histone octamers are separated by 10–11 bp, thereby maintaining the 10 bp ladder generated upon DNase I digestion (Pennings *et al.*, 1991; Ura *et al.*, 1995). Further, given that the high-affinity Fos/Jun binding site is located in the centre of the 180 bp DNA fragment, it is not clear whether this length of DNA would be of sufficient length for long-range sliding. Given that these DNase I footprinting experiments were carried out under identical conditions to that of the mobility gel shift experiments, we conclude that Fos/Jun can disrupt the structure of a nucleosome by altering histone–DNA interactions.

The AP-1 induced disruption of nucleosomal structure is reversible

An important question was whether the observed AP-1 disruption of a nucleosome involved histone displacement from the *fra-2* promoter fragment, or do the histones remain bound to the DNA? To answer this question, an oligonucleotide competition assay was performed (Walter *et al.*, 1995). In this assay, following the formation of an acetylated nucleosome and the subsequent addition of Fos/Jun, a 30 bp oligonucleotide containing an AP-1 binding site was added to the binding reaction. If Fos/Jun caused the displacement of histones from the labelled *fra-2* promoter fragment, the subsequent removal of Fos/Jun by the oligonucleotide would yield protein-free probe DNA being observed by mobility gel shift analysis. An identical result was obtained when a 180 bp oligonucleotide was used as competitor (data not shown). On the other hand, if histones were still present on the *fra-2* promoter fragment, the removal of Fos/Jun could allow the reassembly of the nucleosome. Figure 7 shows that, under conditions where probe DNA was completely assembled into nucleosomes (under these efficient assembly conditions, we often observe a minor, slower-migrating histone–DNA complex of unknown structure), the addition of competitor DNA resulted in the reappearance of the nucleosome (Figure 7, compare lane 7 with lane 3). Alternatively, if an oligonucleotide lacking an AP-1 binding site was used as competitor, the nucleosome did not reappear and Fos/Jun–DNA complexes remained intact (Figure 7, compare lane 11 with lane 3). We therefore can conclude that the observed Fos/Jun disruption of nucleosomal structure is reversible and probably does not involve histone displacement—which is consistent with the observation that this disrupted nucleosomal template does not totally behave like naked DNA in SRY binding experiments (see below).

Fos/Jun can disrupt an acetylated nucleosome to facilitate the subsequent binding of a second transcription factor

As described above, the 180 bp DNA template used here encompasses two high-affinity binding sites for the HMG-box protein SRY (a consensus and non-consensus site, sites 1 and 2 in Figure 8A and B). This is shown, by a

Fig. 7. The disruption of nucleosomal structure caused by Fos/Jun binding is reversible. To acetylated nucleosomes, which were assembled using the N1/N2–(H3,H4) complex and histones H2A/H2B, Fos/Jun (4 fpu) was added (lanes 4–11). Following the binding reaction, increasing concentrations of a 30 bp oligonucleotide that contained (lanes 4–7) or lacked (lanes 8–11) an AP-1 binding site was added. After a 20 min incubation, the nucleoprotein complexes were resolved on a mobility shift gel.

DNase I footprinting experiment, in Figure 8A and is summarized in Figure 8B. Competition experiments have revealed that sites 1 and 2, and the HMG-box binding site TCR α mut 11 (Giese *et al.*, 1992) have almost identical affinities for SRY (data not shown). Interestingly, site 2 encompasses the major start site of transcription. In addition, concerning site 2, a new DNase I-sensitive site (DSS) appeared just 3' of this footprint (arrowed in Figure 8B), which is only observed on naked and not nucleosomal templates (data not shown). Two other lower-affinity HMG-box binding sites were also observed (Figure 8A, sites 3 and 4).

As we demonstrated that Fos/Jun can completely disrupt a nucleosome assembled with the N1/N2–(H3,H4) complex and histones H2A/H2B, a prediction would be that the addition of Fos/Jun first to the nucleosome would disrupt the nucleosome, thereby facilitating the subsequent specific binding of SRY to its recognition sequence. Having characterized the binding of SRY to naked DNA, we employed the footprinting assay to test this prediction. Figure 9 (lanes 3–5) shows that SRY could not bind specifically to an acetylated nucleosome at low protein concentrations (up to 3 fpu) as no clear footprints were observed over sites 1–4 (although a partial footprint covering site 4 could be seen; Figure 9, compare lanes 1 and 5). In complete contrast, if Fos/Jun is added to the acetylated nucleosome prior to the addition of SRY, binding of SRY to site 2 was observed (Figure 9, compare lanes 6–8 with lanes 3–5). The affinity of SRY for this site is identical to that when naked DNA is used as a template, i.e. a complete footprint is observed at 1 fpu. Moreover, the DSS that appeared 3' of site 2 only when the template was naked DNA, also appeared. This indicates that the disrupted nucleosome behaved like a free DNA template.

Binding of SRY to site 1 was not observed because the

Fig. 8. The binding of SRY to naked DNA. **(A)** The binding of increasing concentrations of SRY to the 180 bp *fra-2* DNA fragment was analysed by DNase I footprinting. The rectangles next to the gel illustrate the regions of protection produced by SRY. The appearance of a new DNase I cutting site (DSS) is also shown. **(B)** A summary of the regions protected from DNase I digestion by the binding of Fos/Jun and SRY to *fra-2* DNA. The consensus sequences for SRY (CCATTGT) and AP-1 (TGACTCA) are boxed. Solid lines show regions protected by the proteins, as indicated. Dashed lines indicate the extended region of protection by AP-1 observed in some circumstances. The low-affinity binding site for AP-1 is also shown (dashed line). DSS, DNase I-sensitive site.

Fig. 9. Fos/Jun can disrupt an acetylated nucleosome to facilitate the binding of SRY. Acetylated nucleosomes were assembled using the N1/N2-(H3,H4) complex and histones H2A/H2B. The binding of increasing concentrations of SRY (as shown) in the absence (lanes 3–5) or presence (lanes 6–11) of Fos/Jun to reconstituted nucleosomes was analysed by DNase I footprinting. SRY was added either after (lanes 6–8) or before (lanes 9–11) the addition of 4 fpu of Fos/Jun. Lane 2 received 4 fpu of Fos/Jun without SRY. Lanes 1 and 12 are control lanes. DSS, DNase I-sensitive site.

affinity of SRY for this site is reduced when Fos/Jun occupies its consensus site, since the centre of the two binding sites is separated by 9 bp (however, the reverse is not true; data not shown). Interestingly, binding of SRY

to the lower-affinity sites 3 and 4 is not dramatically enhanced by Fos/Jun binding (Figure 9, compare lanes 6–8 with lanes 3–5). This is consistent with the observation that histones are still present on the *fra-2* DNA fragment and implies that histones may selectively repress the binding of the SRY to low-affinity sites relative to high-affinity sites.

Surprisingly, while the prior binding of Fos/Jun to the reconstituted nucleosome facilitated the binding of SRY to site 2, in complete contrast, when SRY was added first, the binding of Fos/Jun to its recognition site was inhibited (Figure 9, compare lanes 9–11 with lanes 6–8). We have preliminary evidence which indicates that SRY may be binding to an acetylated nucleosome—though not in a sequence-specific manner—and that this binding inhibits the subsequent binding of AP-1. This point is currently under investigation. In summary, the ability of Fos/Jun to destabilize the structure of nucleosome can facilitate the subsequent binding of a second transcription factor, in this case SRY.

Discussion

As an important first step in understanding how bZip proteins interact with a chromatin template, we investigated whether Fos/Jun can interact with a single nucleosome. We have found that Fos/Jun can bind to an unacetylated nucleosome with only a 4- to 5-fold reduction in DNA binding affinity compared with naked DNA. Therefore, Fos/Jun can be classified as a nucleosome-binding transcriptional activator. The novel finding of this work is that when a nucleosome is reconstituted using the physiological N1/N2-(H3,H4) complex, the binding of Fos/Jun to a promoter containing a single high-affinity binding site can cause the complete disruption of the acetylated nucleosome. Moreover, this disruption process could facilitate the subsequent binding of another transcription factor (SRY), which otherwise could not bind, to the DNA template. Taken together, these results suggest that an important function of Fos/Jun, with regard to the transcriptional activation of target genes, may be to remodel chromatin.

The role of histone acetylation in facilitating transcription factor binding

In this study, we have shown that when a nucleosome is assembled using the *Xenopus* N1/N2-(H3,H4) complex plus chicken histones H2A/H2B, Fos/Jun is able to completely disrupt the nucleosome. Both histones H3 and H4 in this complex are acetylated (during *Xenopus* embryogenesis, the diacetylated form of histone H4 is the form of the protein that is incorporated into chromatin during DNA replication; Dimitrov *et al.*, 1993). On the other hand, a ternary complex comprised of Fos/Jun and a nucleosome is observed when principally unacetylated histones H3 and H4 are used in reconstituting the nucleosome. We attribute this difference to the acetylation status of histones H3 and H4.

In contrast to published studies demonstrating that an unacetylated tetramer forms a stable complex (Lee *et al.*, 1993; Walter *et al.*, 1995), the results of Figure 1 demonstrate that, under conditions of the gel mobility shift assay, acetylated histones H3 and H4 do not form a stable

complex with the DNA template. The stabilization of histone H3/H4–DNA interactions and the subsequent formation of a stable nucleosome requires the binding of histones H2A and H2B. Therefore, for Fos/Jun to disrupt the nucleosome may only require the alteration of histone H2A/H2B–(H3/H4)₂ tetramer interactions. In turn, the H3/H4 tetramer is significantly less refractory to the binding of transcription factors (Tremethick *et al.*, 1990; Wolffe, 1991).

Many observations have been reported consistent with the proposal that acetylation of histones H3 and H4 weakens histone–DNA interactions within the nucleosome. For example, acetylated (H3/H4)₂ tetramers elute from a chromatin-bound hydroxyapatite column at lower salt concentrations than do unmodified tetramers (Li *et al.*, 1993), and acetylation of the H4 amino-terminal peptide dramatically reduces its affinity for DNA (Hong *et al.*, 1993). Further, acetylated histones increase the susceptibility of certain cleavage sites within nucleosomal DNA to DNase I (Simpson, 1978) and reduce the linking number per nucleosome (Norton *et al.*, 1990). With regard to function, this potential weakening of histone–DNA interactions caused by acetylation has been shown to facilitate TFI_{II}A binding to *Xenopus* 5S RNA genes within single nucleosomal templates (Lee *et al.*, 1993). In addition, proteolytic removal of N-terminal tails from core histones (which mimics the effect of acetylation) enhances binding of GAL4-AH to a reconstituted nucleosome (Vettese Dadey *et al.*, 1994). It was therefore concluded that the observed inability of GAL4-AH to bind to a nucleosome is mediated primarily by histone amino-terminal tails. Although these data are consistent with our work in terms of acetylation of histones promoting transcription factor binding, neither TFI_{II}A nor GAL-4 binding to a single high-affinity site could significantly disrupt or displace the nucleosome. One possible explanation for this difference is that Fos/Jun—but not TFI_{II}A or GAL-4—has a specific ‘nucleosome disruption’ ability (see below). It is worth pointing out that the assembly of nucleosomes using the *Xenopus* N1/N2–(H3,H4) complex would yield a homogeneous population of acetylated nucleosomes because histones H3 and H4, in the N1/N2 complex, are uniformly acetylated at specific sites, e.g. histone H4 is only acetylated at sites 5 and 12. In contrast, nucleosomes assembled using histones prepared from butyrate-treated HeLa cells would yield a heterogeneous population of acetylated nucleosomes, since histone H4 can be acetylated at all sites (lysines 5, 8, 12 and 16), or only at a subset of these sites in a number of different combinations.

The ability of Fos/Jun to disrupt a nucleosome is dependent on binding to nucleosomal DNA

Based on the following observations, we can conclude that the ability of Fos/Jun to disrupt the nucleosome is absolutely dependent on its ability to interact with DNA and not solely due to protein–protein interactions or any contaminating activities, such as proteolytic enzymes, present in the Fos/Jun preparation. First, a Fos/Jun complex and a histone octamer can coexist on the same DNA template molecule when the Fos/Jun binding site is located at the end of the DNA template where strong histone–DNA interactions do not exist (Figure 3). Second, the ability of Fos/Jun to disrupt a nucleosome is dependent

on its affinity for a particular binding site (Figure 5). Third, when a Fos/Jun titration is carried out using a rotationally positioned nucleosome, the loss of the 10 bp periodicity correlates with the occupancy of the AP-1 binding site. Finally, it is worth noting that, compared with an unpositioned nucleosome, an ~3- to 4-fold higher concentration of Fos/Jun was required to disrupt the rotationally positioned nucleosome. This can be explained by the higher affinity of the histone octamer for the bent DNA template (alternatively, this finding could be related to the orientation of the AP-1 binding site relative to the surface of the histone octamer which is currently being investigated). On the subject of the disruption of this rotationally positioned nucleosome, we can not rule out the possibility that the binding of two Fos/Jun heterodimers is required for this disruption to occur (Figure 6). In general, it would be expected that the number of transcription factor binding sites required to destabilize nucleosomal structure would depend on the affinity of the histone octamer for the DNA (this is particularly relevant given that many nucleosome binding studies employ strongly rotationally positioned nucleosomes).

In addition to the loss of the 10 bp DNase I digestion ladder, the ability of Fos/Jun to disrupt the structure of a nucleosome is also consistent with the observation that (at least under gel mobility shift conditions) the protein–DNA complexes seen when Fos/Jun is added to the acetylated nucleosome run in an identical position to that of Fos/Jun–naked DNA complexes. The SRY and Fos/Jun order of addition experiments (Figure 9) provide further evidence for nucleosomal disruption. The addition of Fos/Jun first to an acetylated nucleosome enabled SRY to bind to the *fra-2* promoter with an affinity similar to that of naked DNA. Interestingly, enhancement of binding to all SRY sites was not observed.

Although the structure of the nucleosome is altered as the result of Fos/Jun binding, histones are not displaced from the DNA template (Figure 7). The ability of histones to rapidly refold into a nucleosome upon removal of Fos/Jun from the nucleoprotein complex provides an attractive mechanism by which an inducible promoter can be rapidly inactivated, in a DNA replication-independent manner, upon cessation of the activation signal.

Chromatin remodelling

Structural studies of specific genes *in vivo* have revealed at least three mechanisms by which nucleosomes can be removed, or their structure altered, from promoter regions: (i) nucleosome exclusion; (ii) nucleosome positioning; and (iii) nucleosome displacement or disruption (Workman and Buchman, 1993; Becker, 1994; Wallrath *et al.*, 1994; Wolffe, 1994).

Strong evidence from biochemical studies and genetic studies in yeast exists showing that, at least in some instances, nucleosome disruption may require the cooperation of a coactivator referred to as the SWI complex (Peterson and Tamkun, 1995). The SWI complex is a conserved multi-subunit complex that uses the energy of ATP to disrupt or displace the nucleosome, thereby facilitating the binding of transcription factors to DNA. Most interestingly, another distinct nucleosome remodelling factor (NURF) has been purified recently from *Drosophila* embryo extracts which may indicate that more than

one mechanism for nucleosome displacement may operate in the eukaryotic nucleus (Tsukiyama and Wu, 1995). Like the SWI complex, NURF requires ATP to alter the structure of a nucleosome (an important question to be addressed is how these remodelling factors are targeted to promoters).

The process of nucleosome displacement may not in all cases require the action of such ATP-dependent coactivators. *In vitro* nucleosome reconstitution experiments have shown that the binding of multiple transcription factors to nucleosomal DNA can weaken histone–histone and histone–DNA interactions sufficiently such that the entire nucleosome can be displaced from template DNA in the presence of histone sinks (Chen *et al.*, 1994; Walter *et al.*, 1995). Similarly, in this study, we have shown that the binding of Fos/Jun to a reconstituted nucleosome can disrupt the nucleosome, which in fact mimics the action of SWI or NURF in many ways.

At present, we do not know the mechanism by which the binding of a Fos/Jun complex to a single high-affinity site can disrupt the structure of a nucleosome. There are however, a number of plausible explanations. As discussed above, we believe that the acetylation status of histones H3/H4 plays an important role. One possibility is that, in combination with the already weakened interactions between DNA and histones due to histone acetylation, if Fos/Jun can bend DNA, the severe bending of DNA produced upon factor binding may not be entirely compatible with the way the DNA is bent around the acetylated histone octamer, thereby further altering the structure of the nucleosome (Kerppola and Curran, 1991b; Kerppola, 1996). This incompatibility may also relate to the change in the conformation of a nucleosome produced by acetylation of histones H3 and H4 (Bauer *et al.*, 1994). Based on this scenario, it would be interesting to investigate the ability of different AP-1 family members to disrupt the nucleosome, since different family members may bend DNA to different extents and in different directions (Kerppola and Curran, 1993). Alternatively, the binding of Fos/Jun may significantly alter the conformation of the nucleosome. Our favoured hypothesis is that Fos/Jun may directly interact via protein interactions with histones (perhaps H2A/H2B) in the nucleosome. If this is the case, this work has narrowed the potential histone target sites to the bZip region of Jun and Fos, plus an acidic region next to the bZip region of Fos. It is this acidic region of Fos that we are currently examining for a histone-binding ability. We also can not eliminate the possibility that more than one Fos/Jun molecule binds to the nucleosome via protein–protein interactions and not via DNA–protein interactions. Finally, since the N1/N2–(H3,H4) preparation is not completely pure, one possibility which we can not rule out at present is that the N1/N2–(H3,H4) complex is contaminated with a component(s) that facilitates the nucleosome disruption process. If such a component exists, we can conclude that it is not SWI or NURF because: (i) the N1/N2–(H3,H4) complex is relatively small (isolated as a 5S complex by sucrose gradient centrifugation) while SWI and NURF are large complexes (2 MDa and 500 kDa, respectively); and (ii) that exogenous ATP, which is absolutely necessary for SWI or NURF to function, is not required for the observed disruption of nucleosomal structure (Figure 2C).

The role of Fos/Jun in chromatin

Fos/Jun, a key member of the AP-1 family, is a sequence-specific transcriptional activator that was found to mediate gene induction by the phorbol ester tumour promoter, TPA. Following this observation, AP-1 activity was found to be induced by a variety of different stimuli including growth factors, cytokines, neurotransmitters, T-cell activators and UV irradiation. Induction of Fos/Jun activity involves different mechanisms which leads to an increase in the abundance of the complex and an induction in its activity (Angel and Karin, 1991; Kerppola and Karin, 1991a; Karin, 1995). Fos/Jun stimulates transcription by binding to promoter regions of target genes. Therefore, like other well-characterized inducible transcription factors, it is likely that Fos/Jun may encounter a repressive chromatin environment. The results of this study strongly suggest that an important function of Fos/Jun, in the complex process that leads to the transcriptional activation of a target gene, is to remodel nucleosomal structure to facilitate the binding of other transcription components. Such a role for AP-1 is supported by the recent finding that a nucleosome located immediately downstream of the transcription start site in the long terminal repeat of the HIV-1 genome is disrupted following TPA treatment (Verdin *et al.*, 1993). This nucleosome encompasses three AP-1 binding sites. Furthermore, demonstrating the importance of acetylation in the AP-1-induced nucleosome disruption process, this positioned nucleosome is disrupted, in the absence of transcriptional activation signals, when histones become hyperacetylated as the result of treatment of cell lines with specific inhibitors of histone deacetylase (Van Lint *et al.*, 1996). Interestingly, like the HIV-1 promoter, the AP-1 binding site of the *fra-2* promoter is also located downstream of the transcription start site. Perhaps disruption of chromatin structure in these promoters is important for the passage of the RNA polymerase II complex. In a different promoter context, transcriptional activators like Fos/Jun, that can bind to a nucleosomal template, may be able to direct the binding of chromatin remodelling factors to appropriate promoters. The subsequent binding of chromatin remodelling factors would in turn cause a more widespread disruption of chromatin structure, which would then allow the functional association of the transcriptional machinery.

Finally, the results of this investigation allow us to put forward a speculative model concerning the molecular steps involved in the conversion of an inactive to an active chromatin structure. Given the ability of Fos/Jun to bind to an unacetylated nucleosome with a high affinity, it is possible that the formation of a potentially active chromatin structure may not in all cases involve nucleosome displacement, but may simply involve the association of a transcriptional activator with a nucleosome(s) on a gene that is going to be transcribed. Displacement of such an activator-bound nucleosome, during the conversion of this potentially active chromatin structure to an active state, could occur as the result of histone modifications, including acetylation and/or phosphorylation. In other words, the nucleosome itself would be the target for signalling pathways. It has been shown that serine residues within the N-terminal region of histone H3 are rapidly phosphorylated during growth induction of quiescent cells (Mahadevan *et al.*, 1991). Similarly, the structure of the nucleosome

could also be modified by the association and modification of non-histone proteins such as high-mobility group proteins 14 and 17 (Tremethick, 1994).

Materials and methods

DNA probes

DNA fragments of 180 bp were generated by PCR from the mouse *fra-2* and rat *fra-1* 5' regulatory sequences, employing the plasmids pTCF2 (Foletta *et al.*, 1994) and pDC12 (D.R.Cohen, unpublished) as the template, respectively. The principal 180 bp *fra-2* DNA fragment used in this investigation contained an AP-1 binding site located next to a HMG-box binding site positioned centrally in the DNA fragment. The sequence of the primers used to generate this fragment were: forward primer 5'-CCC AGC **AAG CTT** GAG TCT AAG GTT GAC (*Hind*III site) and reverse primer 5'- AAA AAG AAT TCT GCG CTC GCT CTC TTT CTC (*Eco*RI site). This DNA fragment encompasses nucleotide position 1562–1744 of the mouse *fra-2* sequence reported by Foletta *et al.* (1994). In Figure 3, the three 180 bp DNA fragments used were generated and labelled by PCR; [α^{32} P]dCTP was included in the reaction mixture. The first of these contained the AP-1 site located in the centre of the DNA fragment (position 1565–1744). The second fragment used contained the AP-1 site located midway between the centre and the 3' end (position 1535–1714). The third DNA fragment contained the centre of the AP-1 site positioned 8 bp away from the 3' end of the fragment. The 180 bp *fra-2* fragment containing an artificial nucleosome positioning sequence was created by following the procedure described by Wechsler *et al.* (1994). Two overlapping single-stranded oligonucleotides (99 nucleotides and 107 nucleotides long) were annealed via their 22 bp overlap and filled in with Klenow DNA polymerase. The sequences of the oligonucleotides were: 5'- CAA GCT TGA GTC TAA GGT TGA CGT GAG TGG ATC CAC ATG GTG CGG GGC TGG AGA ATA AAT AGT GTG GAG TTG AAC TCG TGC **CAT TGT AGT GAC TCA TCT** (99 bases) and 5'- GAA TTC TGC GCT CGC TCT CTT TCT CGT TAC AAG GTC TAA ACC CGA GGT ACA **AGG TCT AAA CCC GAG AGC CCC TGC GCT CTG CCC GAG ATG AGT CAC TAC AAT GGC AC** (107 bases). The AP-1 and HMG-box binding sites are shown in bold, and the nucleosome positioning sequence, a tandem repeat of the 20-bp GT sequence (Shrader and Crothers, 1989), is underlined. The *fra-1* DNA fragment used in Figure 5 contains two AP-1-like binding sites (sites 1 and 2) that are separated by 57 bp. Site 1 is located 54 bp from the 5' end and site 2 is located 55 bp from the 3' end. The sequences of the primers used were: forward primer 5'- CTT GGG **AAG CTT** GAC TAG TCT GGA CGG GGC GGG (*Hind*III site) and reverse primer 5'- AAA AAG AAT TCA GCG ACC GAC TTG GCG TCC CCT (*Eco*RI site). The DNA fragments were cut with the appropriate restriction enzyme, end-labelled with Klenow DNA polymerase and purified on a 1× Tris–borate EDTA (TBE) polyacrylamide gel (8%).

Transcription factor purification

Fos, Fos (116–211), Jun and Jun (224–232), tagged at their N-terminal ends with six histidine residues (6×His), were prepared and purified by affinity chromatography using a Ni–NTA–agarose column (Qiagen) as described previously (Abate *et al.*, 1990). Fos or Jun proteins were refolded by step-wise dialysis with the final buffer containing 25 mM sodium phosphate pH 7.6, 5% glycerol and 5 mM DTT. Full-length human 6×His-SRY was prepared as described by Cohen *et al.* (1994). A fragment encompassing the HMG-box of SRY and including a 6×His tag was generated by PCR, using pCMV-SRY (Cohen *et al.*, 1994) as the template, and this fragment was ligated into the expression vector pDS56 and sequenced to confirm its integrity. Following elution from a Ni–NTA–agarose column, proteins were dialysed against 25 mM sodium phosphate, pH 7.4, 10% glycerol, 5 mM MgCl₂ and 1 mM DTT.

Nucleosome assembly

The N1/N2–(H3,H4) complex was isolated from *Xenopus laevis* ovaries, histone H2A/H2B dimers were purified from chicken red blood cells, and the assembly of nucleosomes was carried out essentially as described by Tremethick and Frommer (1992). The standard nucleosome assembly mixture (10 µl) consisted of N1/N2–(H3,H4) complex, H2A/H2B dimer, 100 ng of non-specific carrier DNA (a 180 bp DNA fragment), and 1 ng of labelled DNA probe. The assembly reaction also contained 50 mM NaCl, 1.5 mM MgCl₂, 15 mM HEPES, pH 7.5, 3 mM ATP, 0.1 mM EDTA and 1 mM DTT (3 mM ATP can be replaced with 2.4 mM

EDTA). All incubations were carried out at 27°C for 4 h. Nucleosome core reconstitution, using chicken H1-depleted oligonucleosomes as donor chromatin and the 180 bp DNA probe as the recipient, was carried out as described by Adams and Workman (1995). After the final dilution step, 2.5 mM MgCl₂ was added to precipitate the donor chromatin (17). The aggregated chromatin was then removed by a 30 min spin in a microcentrifuge at 4°C (Schwarz and Hansen, 1994).

Binding reactions

Purified Fos and Jun proteins, in a final volume of 10 µl, were mixed and incubated at 37°C for 15 min in 25 mM sodium phosphate, pH 7.6, 5% glycerol and 5 mM DTT. Following this, in a volume of 5 µl, 1 µg of poly [dI.dC] (Pharmacia), 1 mg/ml bovine serum albumin, and binding mix (producing final concentrations of 16.7 mM Tris–HCl, pH 7.5, 1.7 mM MgCl₂, 0.17% Nonidet P-40, 1.7 mM EDTA, 8 mM DTT, 4.6% glycerol and 4.8% sucrose) were added and the mixture was allowed to incubate for 10 min at 24°C. 10 µl of reconstituted nucleosomes (assembled from chicken chromatin or from the N1/N2–(H3,H4) complex, or mock reactions), were then added to the Fos/Jun mixture. The binding reaction was allowed to proceed for 20 min at 24°C. The final binding conditions, in a total volume of 25 µl (for mock-assembled and both types of nucleosome-assembled templates), contained 50 mM NaCl, 10 mM Tris–HCl, pH 7.5, 1.5 mM MgCl₂, 5 mM DTT, 1 mM EDTA, 5% glycerol, 5% sucrose, 0.1% Nonidet P-40, 0.6 mg/ml bovine serum albumin, 5 mM HEPES, pH 7.5, and 10 mM sodium phosphate, pH 7.6. Identical binding conditions were used for SRY and the HMG-box domain of SRY. Because of the variability in binding activity between different preparations of recombinant transcription factors, we expressed the amount of Fos/Jun and SRY in terms of footprinting units (fpu). For Fos/Jun, 1 fpu is equivalent to the amount of Fos/Jun needed to produce a complete footprint, on naked DNA, at the AP-1 consensus site present in the *fra-2* promoter. Similarly, for SRY, 1 fpu is equivalent to the amount of protein needed to produce a complete footprint, on naked DNA, at site 1 (see Figure 8A) present in the *fra-2* promoter.

Gel mobility shift assays and DNase I footprinting

For gel mobility shift assays, binding reactions received loading dye, were loaded directly onto a 4.5% polyacrylamide gel (acrylamide: bisacrylamide, 29:1)–0.5×TBE gel, and run at 200 V for 3 h at 4°C. For DNase I footprinting, following the addition of 2.5 µl of a 10 mM MgCl₂/5 mM CaCl₂ solution and a 1 min incubation at 24°C, binding reaction mixtures were digested with DNase I (Boehringer-Mannheim). Reconstituted templates and mock-assembled templates were digested for 1 min at 24°C with 1.8 and 0.2 U of DNase I, respectively. Digestion was terminated by the addition of 70 µl of 200 mM sodium acetate, 30 mM EDTA, 0.2% SDS and 100 mg/ml tRNA (Sigma). Following purification of the DNA, the samples were resuspended in 3 µl of loading dye (deionized formamide containing 10 mM EDTA, 0.3% bromophenol blue and 0.3% xylene cyanol), heated at 80°C for 2 min, placed immediately into an ice-bath, and then loaded directly onto a pre-run 8% acrylamide–7 M urea denaturing gel. The samples were resolved at 1500 V for 2 h. For denaturing and non-denaturing gels, gels were transferred to a Whatman DEAE ion-exchange paper, dried under vacuum and exposed to X-ray film (Kodak) at room temperature.

References

- Abate,C., Luk,D., Gagne,E., Roeder,R.G. and Curran,T. (1990) Fos and jun cooperate in transcriptional regulation via heterologous activation domains. *Mol. Cell. Biol.*, **10**, 5532–5535.
- Adams,C.C. and Workman,J.L. (1995) Binding of disparate transcriptional activators to nucleosomal DNA is inherently cooperative. *Mol. Cell. Biol.*, **15**, 1405–1421.
- Angel,P. and Karin,M. (1991) The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochim. Biophys. Acta*, **1072**, 129–157.
- Archer,T.K., Cordingley,M.G., Wolford,R.G. and Hager,G.L. (1991) Transcription factor access is mediated by accurately positioned nucleosomes on the mouse mammary tumor virus promoter. *Mol. Cell. Biol.*, **11**, 688–698.
- Bauer,W.R., Hayes,J.J., White,J.H. and Wolffe,A.P. (1994) Nucleosome structural changes due to acetylation. *J. Mol. Biol.*, **236**, 685–690.
- Becker,P.B. (1994) The establishment of active promoters in chromatin. *BioEssays*, **16**, 541–547.

- Bergers, G., Graninger, P., Braselmann, S., Wrighton, C. and Busslinger, M. (1995) Transcriptional activation of the fra-1 gene by AP-1 is mediated by regulatory sequences in the first intron. *Mol. Cell. Biol.*, **15**, 3748–3758.
- Berta, P., Hawkins, J.R., Sinclair, A.H., Taylor, A., Griffiths, B.L., Goodfellow, P.N. and Fellous, M. (1990) Genetic evidence equating SRY and the testis-determining factor. *Nature*, **348**, 448–450.
- Blomquist, P., Li, Q. and Wrangé, O. (1996) The affinity of nuclear factor 1 for its DNA site is drastically reduced by nucleosome organization irrespective of its rotational or translational position. *J. Biol. Chem.*, **271**, 153–159.
- Bouvet, P., Dimitrov, S. and Wolffe, A.P. (1994) Specific regulation of *Xenopus* chromosomal 5S rRNA gene transcription *in vivo* by histone H1. *Genes Dev.*, **8**, 1147–1159.
- Chen, H., Li, B. and Workman, J.L. (1994) A histone-binding protein, nucleoplasmin, stimulates transcription factor binding to nucleosomes and factor-induced nucleosome disassembly. *EMBO J.*, **13**, 380–390.
- Cohen, R.D., Sinclair, A.H. and McGovern, J.D. (1994) SRY protein enhances transcription of Fos-related antigen 1 promoter constructs. *Proc. Natl Acad. Sci. USA*, **91**, 4372–4376.
- Dimitrov, S., Almouzni, G., Dasso, M. and Wolffe, A.P. (1993) Chromatin transitions during early *Xenopus* embryogenesis: changes in histone H4 acetylation and in linker histone type. *Dev. Biol.*, **160**, 214–227.
- Foletta, V.C., Sonobe, M.H., Suzuki, T., Endo, T., Iba, H. and Cohen, D.R. (1994) Cloning and characterisation of the mouse fra-2 gene. *Oncogene*, **9**, 3305–3311.
- Giese, K., Cox, J. and Grosschedl, R. (1992) The HMG domain of lymphoid enhancer factor 1 bends DNA and facilitates assembly of functional nucleoprotein structures. *Cell*, **69**, 185–195.
- Glover, J.N.M. and Harrison, S.C. (1995) Crystal structure of the heterodimeric bZIP transcription factor c-Fos-c-Jun bound to DNA. *Nature*, **373**, 257–261.
- Hong, L., Schroth, G.P., Matthews, H.R., Yau, P. and Bradbury, E.M. (1993) Studies of the DNA binding properties of histone H4 amino terminus. *J. Biol. Chem.*, **268**, 305–314.
- Kamakaka, R.T., Bulger, M. and Kadonaga, J.T. (1993) Potentiation of RNA polymerase II transcription by Gal4-VP16 during but not after DNA replication and chromatin assembly. *Genes Dev.*, **7**, 1779–1795.
- Karin, M. (1995) The regulation of AP-1 activity by mitogen-activated protein kinases. *J. Biol. Chem.*, **270**, 16483–16486.
- Kerppola, T.K. (1996) Fos and Jun bend the AP-1 site: effects of probe geometry on the detection of protein-induced DNA bending. *Proc. Natl Acad. Sci. USA*, **93**, 10117–10122.
- Kerppola, T.K. and Curran, T. (1991a) Transcription factor interactions: basics on zippers. *Curr. Opin. Struct. Biol.*, **1**, 71–79.
- Kerppola, T.K. and Curran, T. (1991b) Fos-Jun heterodimers and Jun homodimers bend DNA in opposite orientations: implications for transcription factor cooperativity. *Cell*, **66**, 317–326.
- Kerppola, T.K. and Curran, T. (1993) Selective DNA bending by a variety of bZIP proteins. *Mol. Cell. Biol.*, **13**, 5479–5489.
- Lee, D.Y., Hayes, J.J., Pruss, D. and Wolffe, A.P. (1993) A positive role for histone acetylation in transcription factor access to nucleosomal DNA. *Cell*, **72**, 73–84.
- Li, B., Adams, C.C. and Workman, J.L. (1994) Nucleosome binding by the constitutive transcription factor Sp1. *J. Biol. Chem.*, **269**, 7756–7763.
- Li, Q. and Wrangé, O. (1995) Accessibility of a glucocorticoid response element in a nucleosome depends on its rotational positioning. *Mol. Cell. Biol.*, **15**, 4375–4384.
- Li, W., Nagaraja, S., Delcuve, G.P., Hendzel, M.J. and Davie, J.R. (1993) Effects of histone acetylation, ubiquitination and variants on nucleosome stability. *Biochem. J.*, **296**, 737–744.
- Mahadevan, L.C., Willis, A.C. and Barratt, M.J. (1991) Rapid histone H3 phosphorylation in response to growth factors, phorbol esters, okadaic acid, and protein synthesis inhibitors. *Cell*, **65**, 775–783.
- Norton, V.G., Marvin, K.W., Yau, P. and Bradbury, E.M. (1990) Nucleosome linking number change controlled by acetylation of histones H3 and H4. *J. Biol. Chem.*, **265**, 19848–19852.
- Pennings, S., Meersseman, G. and Bradbury, E.M. (1991) Mobility of positioned nucleosomes on 5S rDNA. *J. Mol. Biol.*, **220**, 101–110.
- Peterson, C.L. and Tamkun, J.W. (1995) The SWI-SNF complex: a chromatin remodeling machine? *Trends Biochem. Sci.*, **20**, 143–146.
- Pruss, D. and Wolffe, A.P. (1993) Histone-DNA contacts in a nucleosome core containing a *Xenopus* 5S rRNA gene. *Biochemistry*, **32**, 6810–6814.
- Schwarz, P.M. and Hansen, J.C. (1994) Formation and stability of higher order chromatin structures. *J. Biol. Chem.*, **269**, 16284–16289.
- Shrader, T.E. and Crothers, D.M. (1989) Artificial nucleosome positioning sequences. *Proc. Natl Acad. Sci. USA*, **86**, 7418–7422.
- Simpson, R.T. (1978) Structure of chromatin containing extensively acetylated H3 and H4. *Cell*, **13**, 691–699.
- Taylor, I.C., Workman, J.L., Schuetz, T.J. and Kingston, R.E. (1991) Facilitated binding of GAL4 and heat shock factor to nucleosomal templates: differential function of DNA-binding domains. *Genes Dev.*, **5**, 1285–1298.
- Tremethick, D.J. (1994) High mobility group proteins 14 and 17 can space nucleosomal particles deficient in histones H2A and H2B creating a template that is transcriptionally active. *J. Biol. Chem.*, **269**, 28436–28442.
- Tremethick, D.J. (1996) High mobility group proteins 14 and 17 can prevent the close packing of nucleosomes by increasing the strength of protein contacts in the linker DNA. *J. Biol. Chem.*, **271**, 12009–12016.
- Tremethick, D.J. and Drew, H.R. (1993) High mobility group proteins 14 and 17 can space nucleosomes *in vitro*. *J. Biol. Chem.*, **268**, 11389–11393.
- Tremethick, D.J. and Frommer, M. (1992) Partial purification, from *Xenopus laevis* oocytes, of an ATP-dependent activity required for nucleosome spacing *in vitro*. *J. Biol. Chem.*, **267**, 15041–15048.
- Tremethick, D., Zucker, K. and Worcel, A. (1990) The transcription complex of the 5S RNA gene, but not transcription factor IIIA alone, prevents nucleosomal repression of transcription. *J. Biol. Chem.*, **265**, 5014–5023.
- Tsukiyama, T. and Wu, C. (1995) Purification and properties of an ATP-dependent nucleosome remodeling factor. *Cell*, **83**, 1011–1020.
- Ura, K., Hayes, J. and Wolffe, A.P. (1995) Positive role for nucleosome mobility in the transcriptional activity of chromatin templates: restriction by linker histones. *EMBO J.*, **14**, 3752–3765.
- Van Lint, C., Emiliani, S., Ott, M. and Verdin, E. (1996) Transcriptional activation and chromatin remodeling of the HIV-1 promoter in response to histone acetylation. *EMBO J.*, **15**, 1112–1120.
- Verdin, E., Paras, P., Jr and Van Lint, C. (1993) Chromatin disruption in the promoter of human immunodeficiency virus type 1 during transcriptional activation. *EMBO J.*, **12**, 3249–3259.
- Vettese Dadey, M., Walter, P., Chen, H., Juan, L.J. and Workman, J.L. (1994) Role of the histone amino termini in facilitated binding of a transcription factor, GAL4-AH, to nucleosome cores. *Mol. Cell. Biol.*, **14**, 970–981.
- Wallrath, L.L., Lu, Q., Granok, H. and Elgin, S.C. (1994) Architectural variations of inducible eukaryotic promoters: preset and remodeling chromatin structures. *BioEssays*, **16**, 165–170.
- Walter, P.P., Owen Hughes, T.A., Cote, J. and Workman, J.L. (1995) Stimulation of transcription factor binding and histone displacement by nucleosome assembly protein 1 and nucleoplasmin requires disruption of the histone octamer. *Mol. Cell. Biol.*, **15**, 6178–6187.
- Wechsler, D.S., Papoulas, O., Dang, C.V. and Kingston, R.E. (1994) Differential binding of c-Myc and Max to nucleosomal DNA. *Mol. Cell. Biol.*, **14**, 4097–4107.
- Weischet, W.O., Allen, J.R., Riedel, G. and Van Holde, K.E. (1979) The effects of salt concentration and H1 depletion on the digestion of calf thymus chromatin by micrococcal nuclease. *Nucleic Acids Res.*, **6**, 1843–1862.
- Wolffe, A.P. (1991) Implications of DNA replication for eukaryotic gene expression. *J. Cell Sci.*, **99**, 201–206.
- Wolffe, A.P. (1994) Transcription: in tune with the histones. *Cell*, **77**, 13–16.
- Workman, J.L. and Buchman, A.R. (1993) Multiple functions of nucleosomes and regulatory factors in transcription. *Trends Biochem. Sci.*, **18**, 90–95.
- Workman, J.L., Taylor, I.C. and Kingston, R.E. (1991) Activation domains of stably bound GAL4 derivatives alleviate repression of promoters by nucleosomes. *Cell*, **64**, 533–544.
- Zucker, K. and Worcel, A. (1990) The histone H3/H4.N1 complex supplemented with histone H2A-H2B dimers and DNA topoisomerase I forms nucleosomes on circular DNA under physiological conditions. *J. Biol. Chem.*, **265**, 14487–14496.

Received on May 20, 1996; revised on December 13, 1996