© 1991 Oxford University Press

DNA protein interactions at the interferon-responsive promoter elements: potential for an H-DNA conformation

Christian Roy and Bernard Lebleu*
Laboratoire de Biochimie des Protéines, URA CNRS 1191, Université Montpellier II, Place E. Bataillon CP012, 34095 Montpellier Cedex 5, France

Received November 6, 1990; Revised and Accepted January 7, 1991

ABSTRACT

The regions of several genes (IFI-56K, HLA-A3, HLA-DR and 6–16) containing the (putative) ISRE (Interferon Stimulatable Response Element) were tested for their ability to be recognized by HeLa cells nuclear extract proteins. In a band shift assay, all probes yielded two B1 and B2 DNA-protein complexes of similar mobilities. Unexpectedly the titration of the B1 complex with a synthetic ISRE core (OL1), promoted the formation of B2. Both the probe and OL1 were recovered in B2. For each probe, the possibility of the part of the sequence involved in B1 complex to form a H-DNA structure with the part of the sequence involved in B2 exists. Such a structure was favored by the colinearity of the pairing regions and requires ATP. Although probes seemed to have a secundary structure, the formal existence of a H-DNA structure has not been demonstrated.Such a model could be extended to other interferon inducible gene promoters and may account for their binding properties and differential inducibility after 5′ deletion or point mutations.

INTRODUCTION

IFNs are glycosylated peptides which exhibit antiviral, antiproliferative and immunomodulatory properties (1–3). They interact with a membranous receptor specific of either type I ($\alpha\beta$) or type II (γ) IFN (4–9). This interaction leads to the activation of a latent cytoplasmic DNA binding protein which then migrates to the nucleus within minutes (10,11), and to the subsequent activation of several genes. IFN α and IFN γ do not induce identical sets of genes (3,12,13). Moreover, the signalling pathways are not the same, which may lead to synergistic activation of some genes (14–17). On the basis of sequence homology, a potential regulatory cis acting element of 30 base pairs (ICS or Interferon Consensus Sequence) has been defined by Friedman and Stark (18). This element was found in genes inducible by either types of IFNs at various distances upstream (19–27) or downstream (21,28) of the transcription initiation site, either alone or repeated (21,25,28) in any orientation. Further characterization of the IFN system led to the cloning of a number of additional inducible genes and the initial consensus became restricted to 12–13 base pairs. The ISRE or IFN Stimulatable Responsive Element was defined on the basis of its ability to confer IFN sensitivity to a reporter gene. This sequence is recognized by different proteins as shown in several cell systems; IFN treatment increases the number of proteins interacting with this consensus sequence or their relative abundance (23,25,26,29,30). Responses differ according to the species of IFN used, the gene under study and the cell type.

Our initial work along these lines showed that a synthetic oligonucleotide representing the ICS could be recognized by proteins in a nuclear extract prepared from HeLa cells and that the binding activity was increased upon IFN addition ($\alpha\beta$ or γ) to the cells (31). A subsequent study showed that probes containing the ICS or putative ISRE of the HLA-A3, HLA-DR and IFI-56K genes could be retarded in two bands in a band shift assay (32). This was also the case for the 6–16 ISRE (33 and this paper) which was taken as an additional probe because of its extensive homology with the ISRE of other IFN-inducible genes (25,26,29,34). The lowest mobility band (B2) was present essentially in nuclear extracts originating from IFN-treated cells. This binding was ATP-dependent and this sensitivity to ATP appeared to be the basis of IFN action suggesting the potential role of regulatory kinases (32).

The present work aimed at further characterizing these observations mainly because 1) all the probes used elicited identical gel band shift patterns and 2) DNase I action on the complexes revealed similar patterns of digestion although only one portion of the probes (that containing the ICS) had a high sequence homology. A double-stranded oligonucleotide (OL1) representing the part of the ICS defining the common interacting sequence was added as a competitor for the ICS containing region of the probes in order to further characterize the B2 complex formed with the other end of the probes. Surprisingly, titration of B1 with OL1 favored the detection of the slow migrating species (B2). Moreover, both OL1 and the ICS-containing probe were recovered in B2. The B1 complex was part of the B2 one. To account for these results, we raise the possibility that a portion of the probe is able to fold back on its other end in a way that is predicted for triple helix formation (35). This leads to the potential existence of a H-DNA conformation. This hypothesis is satisfactory for the 4 probes tested. Such results emphasize

* To whom correspondence should be addressed

the potential role of element(s) cis to a consensus sequence and the structural information latent in a DNA sequence beyond strict homologies.

MATERIALS AND METHODS

Cell culture

For nuclear extracts preparation, HeLa cells were grown in suspension in MEM medium supplemented with 10% (v/v) fetal calf serum. Cells were used at a density of 0.8×10^6 cells/ ml. Nuclear extracts were prepared according to Dignam et al. (36). Type I ($\alpha\beta$) IFN originating from Namalva cells (1.2×10^6 IU/ mg) was donated by Merieux (Lyon, France). Cells were treated with 500 U/ml IFN for 1 h. Proteins were assayed according to Bradford (37).

Oligonucleotides

Oligonucleotides were purified by gel electrophoresis (12% (w/v) acrylamide-8.3 M urea) and purified on Sep-Pak cartridge (Waters). Oligonucleotides were labeled using (α-^{32}P) ddATP (3000 Ci/mM) with terminal transferase (Pharmacia). Each strand was hybridized to the other in 500 mM NaCl by boiling for 2 min and then slowly lowering the temperature to 42°C. The unincorporated ddATP was eliminated with a Nick Column (Pharmacia).

Band shift assay

Nuclear extracts were preincubated at 30°C under 30 μl in a medium containing 20 mM Hepes-KOH pH 7.9, 10% (v/v) glycerol, 0.25 mM dithiothreitol, 0.1 mM EDTA, 50 mM KCl, 0.5 mM ATP, 2 mM $MgCl_2$, 0.5 μg E. Coli DNA, 0.5 μg poly (dI) poly (dC) and nuclear proteins as indicated. Then $12-30 \times 10^3$ cpm of the probe (0.03–0.1 ng) were added and the incubation continued for 10 min. Bound and free probes (20 μl) were separated on gels (acrylamide/bisacrylamide: 60/1) containing 6% (v/v) glycerol, 10 mM Tris-HCl pH 7.4 and 2 mM $MgCl_2$. The gels were prerun at 4°C for 2h at 10 volts/cm using 10 mM Tris HCl pH 7.4 and 2 mM $MgCl_2$ as a running buffer.

DNase I footprinting experiments

Incubations were performed as described above with amounts of nuclear extract proteins as specified in the legends. Samples were then incubated for 3 min at 30°C with DNase I (Sigma)(for amounts, see legends) and processed for analysis on a sequencing gel (12% acrylamide-8.3 M urea). If after DNase I action the samples were subjected to a band shift assay, the gels were transferred for 45 min in 0.5× TBE on DEAE paper. After autoradiography, the bands corresponding to the bound and free probes were eluted in 1.25 M NaCl overnight at 42°C. Samples were extracted with phenol, precipitated with ethanol and analyzed by gel electrophoresis.

TABLE I: Probe sequences and location.

GENE		location
IFI-56K	5'TTCTATTTTAAAACAGATAAACTGCAGACCTGCAACCATAGATGTTTCTA 3'AAGATAAAATTTTGTCTATTTGACGTCTGGACGTTGGTATCTACAAAGAT	-219/-170
HLA-DR	5'AGAGAAAATGAGAGGAGAAACTCTGAGGTTCAACACTCTCAACACTGGAG 3'TCTCTTTTACTCTCCTCTTTGAGACTCCAAGTTGTGAGAGTTGTGACCTC	-557/-606
HLA-A3	5'GAGCAGGGGAGAAAAGAAACTGCGGAGTTGGGAATCCCAAGGCTGGGAC 3'CTCGTCCCCTCTTTTCTTTGACGCCTCAACCCCTTAGGGTTCCGACCCTG	*
6-16	5'GAGCTGGGAGAGAGGGGAAAATGAAACTGCAGAGTGCAC 3'CTCGACCCTCTCTCCCCTTTTACTTTGACGTCTCACGTG	-126/-88
ICS	5'AGTAGAGGAGAAACTGCGGAGGTGGTGAA 3'TCATCTCCTCTTTGACGCCTCCACCACTT	
OL1	5'AGTAGAGGAGAAAC 3'TCATCTCCTCTTTG	
VSV	5'TGAAAAAAACTAACAG 3'ACTTTTTTTGATTGTC	intergenic region

The location in the gene of the probes used is indicated in the right part of the Table. ICS has been previously described (31). OL1 represents the left portion of the ICS. The probe identified as VSV corresponds to the vesicular stomatitis virus intergenic region (the upper strand being the negative strand). The core motif AAACT/TTTGA is boxed for all the probes and the probes lined up around this core. Every 10th base is underlined. Triangles represent cutting sites for restriction enzymes (Hinf I for HLA-A3, Pst I for both 6–16 ISRE and IFI-56K). The underlined part of the sequences are those which are involved in a potential H-DNA structure, the left part of the sequence folding back to the right part (line above the sequences).

* The cap site of this gene has not been determined.

RESULTS

Protection from DNase I action of the probes both at the level of the ICS and cis to the ICS

As already described (31), the ICS-containing sequences (Table I) were retarded in two bands, B1 and B2, B2 having the lowest mobility. Detection of the B2 complex was highly dependent on the concentration of the nuclear extract during the binding reaction and on the pretreatment of the cells by $\alpha\beta$ or γ IFN. All the experiments reported in this paper were done with nuclear extracts originating from $\alpha\beta$ IFN-treated HeLa cells. For all the probes used, the mobility of the two bands was the same, each probe being able to compete for binding with any of the others. Most experiments are presented using the HLA-A3 probe but were carried out with the four probes listed in Table I with only qualitative differences.

The data presented in Fig.1 show the DNase I footprinting pattern of the probes. The following points should be noted: 1) depending on the dose of nuclear extract, one or two regions were protected; 2) the sensitivity to DNase I of these regions was different, the region at the 3′ end of the AAACT (dots) motif being more sensitive to DNase I action; 3) at the lowest protein concentration, the protection of this latter region was less or not detectable at all; 4) the patterns obtained with each probe resemble each other, in line with an homology at their 5′ end but not with the lack of similitude at their 3′ end (see below). A similar pattern but with a lower definition was observed for the complementary strands of the probes (not shown). Since only B1 was detected in the band shift assay at low protein concentration (32 and below), the DNase I protected region bordered at its 3′ end by the AAACT motif must correspond to the part of the probe involved in the B1 complex.

These observations could be accounted for as follows: (i) the binding of the second (protein) factor is dependent on the binding of the first one, or (ii) its amount and/or its relative affinity is lower so that its binding occurs after the binding of the first one. In no case did binding on the two portions of the probes appear mutually exclusive.

Titration of the ICS-binding protein (B1) promoted the formation of a lower mobility complex (B2)

To distinguish between these two possibilities, we used a probe representing the ICS (see Table I) This probe forms a complex analogous to B1 in the presence of nuclear extracts and competes for binding with the 50 mer probes (32). In addition, DNase I

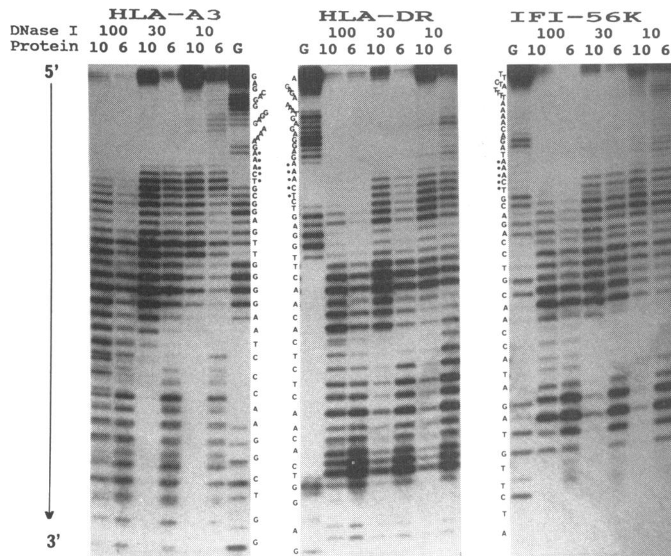

Fig. 1: Differential protection of two regions of the probes from DNase I action: effects of nuclear protein extract amount and DNase I concentration. The AAACT (dots) containing strand was labeled (upper strand in Table I). Amounts of nuclear extracts from $\alpha\beta$ IFN treated cells were either 10 or 6 μg (i.e. 0.33 or 0.2 mg protein/ml). Most of the probes was bound even at the lowest protein concentration. DNase I was added at the end of the incubation at 100, 30 or 10 $\mu g/ml$ and allowed to act for 3 min. at 30°C. When, together with the labeled probe, an excess of unlabeled probe was added, such as no specific binding could be detected in the band shift assay, the probe was degraded upon DNase I action and a ladder, not extending more than 10–15 nucleotides, was detectable (not shown). Lane G: piperidine cleavage of pyridinium formiate-treated probes.

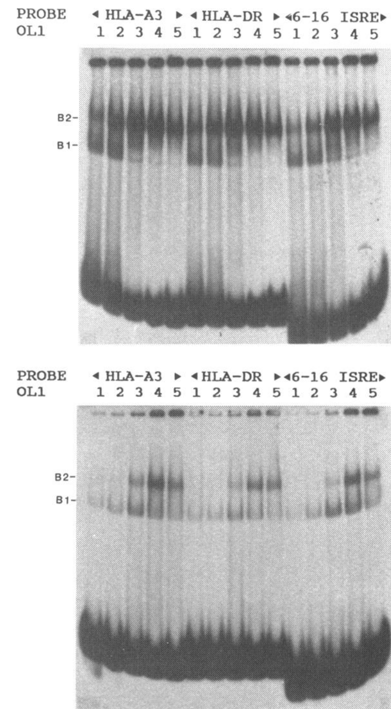

Fig. 2: Ability of OL1 to promote B2 formation. The probes used in this band shift assay are listed at the top of the Fig. The doses of OL1 were: 1 : none; 2 : 4 nM; 3 : 20 nM; 4 : 100 nM and 5 : 500 nM. Upper panel : 5 μg nuclear proteins, lower panel : 3 μg.

footprinting reveals protection in a region homologous to that described for B1 in the 50 mer probes. A double stranded oligonucleotide representing the 5′ part of the ICS (OL1 in Table I) was synthetized as a competitor for the formation of B1. OL1 was able to form a complex whose mobility was similar to that of B1 (not shown). This complex was rather unstable since the signal could only be detected when the exposure time or probe amount was increased 10 fold.

Two experimental conditions were chosen: a low protein concentration (Fig. 2, bottom) where only the B1 complex should be detected and a higher one (Fig.2, top) where both complexes should be formed. At the highest protein concentration, increasing the OL1 concentration led to a gradual loss of the B1 complex as expected from the sequence homology between OL1 and the region which is bordered at its 3′ end by the AAACT motif in the 50 mer probes. At low protein concentration, in the absence of OL1, only B1 was detectable (lane 1)(32). Increasing the OL1 concentration surprisingly led to the appearance of B2. This suggests that B2 results from the interaction of at least two proteins with different parts of the probes and that B2 depends on the prior existence of B1.

B2 complex contained both OL1 and the 50-mer probe.

Direct evidence that both OL1 and the 50 mer probe were involved in the same complex was obtained by performing a band shift assay (Fig.3) using both radiolabeled OL1 and 50 mer probes under conditions similar to those described in Fig. 2. The ratio of c.p.m. of OL1 to 50 mer probes was adjusted to take into account their relative affinity. After gel migration and autoradiography, both B1 and B2 were excised and their DNA content analysed. When each of the radiolabeled probes was used alone, OL1 was recovered within the B1 band (lane D) and the 50 mer probes within both B1 and B2 (at least at high protein concentration, lanes C and E). When both radiolabeled probes were added together (lanes A,B,F and G), OL1 was still recovered in B1 as well as various amounts of the 50 mer probes depending on the protein concentration. Surprisingly, both probes were also systematically detected within B2 (lanes A,F and G). The molar ratio of OL1 and 50-mer probe in these B2 complexes could not be determined.

B1 and B2 complexes were not mutually exclusive

To understand the mechanisms leading to B2 formation, it had to be demonstrated whether the formation of B1 was a prerequisite for the existence of B2 and whether or not B1 was part of B2. Albeit OL1 addition greatly facilitated the appearance of B2, the B2 complex formed in the presence of OL1 was highly susceptible to DNase I digestion. This might be due to a lower affinity of DNA binding protein(s) for OL1 or to the destabilization of the B2 complex. Attempts to stabilize such a structure were performed using BS^3 (Bis(sulfosuccinimidyl) suberate) a protein cross-linking reagent (Fig. 4). The following points should be noted : 1) the migration of the probe alone was not affected by BS^3 addition (lane 1); 2) the addition of unlabeled probe in excess did not demonstrated any retarded band with BS^3 (lane 7); 3) the addition of BS^3 allowed the appearance of a previously undetected complex of low mobility (B3); 4) the amount of B3 was dependent on the dose of BS^3; 5) B3 increased to the

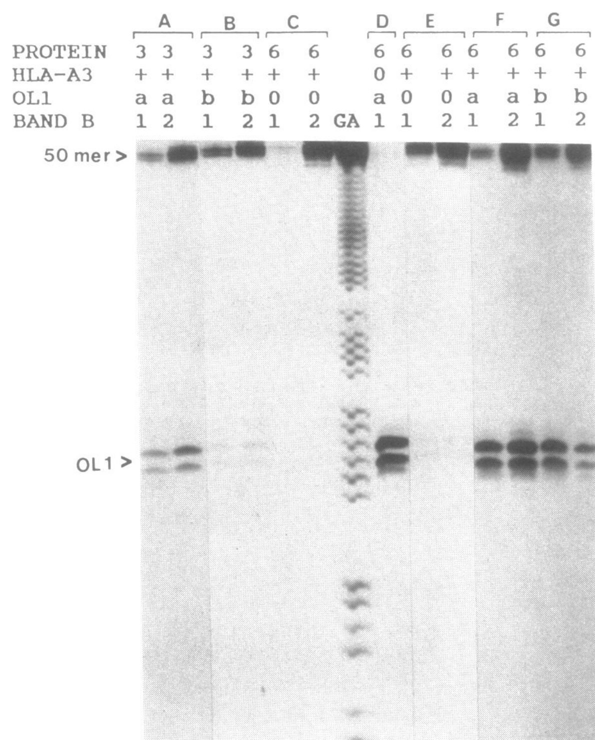

Fig. 3: Recovery of both HLA-A3 probe and OL1 in B2 complex. Incubations were carried out in the presence of 2×10^4 c.p.m. of 50 mer probe and 3×10^5 (a) or 6×10^4 (b) c.p.m. OL1 when indicated. Nuclear protein amounts indicated are in μg. After gel retardation, the bands corresponding to B1 (1) or B2 (2) were excised and analyzed for their probe content in a 15% (w/v) acrylamide/urea sequencing gel. The GA lane corresponds to piperidine cleavage after pyridinium formiate treatment of the probe and allows the determination of the size of the retarded probes. Lanes C and E correspond to identical conditions. The two bands observed at the OL1 level could correspond either to a mixture of 13 and 14 mer after incomplete OL1 purification or to strand separation after denaturating electrophoresis (note the highly assymetric base composition of the two strands of OL1).

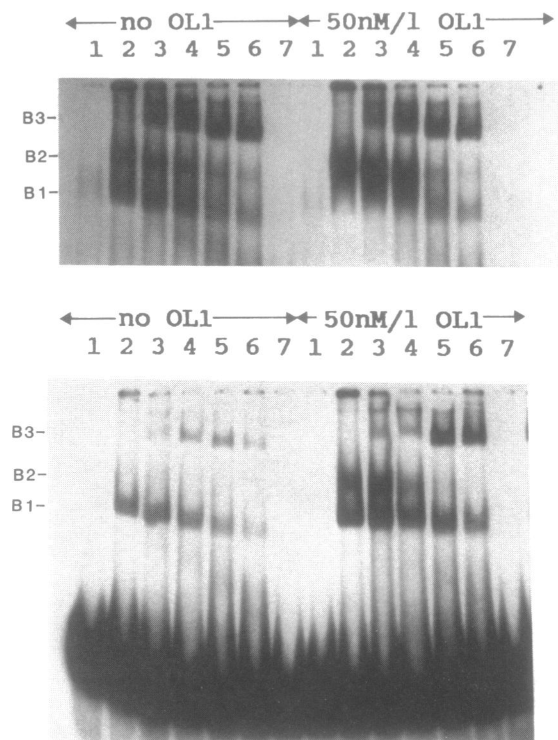

Fig. 4: Stabilization of protein/DNA interactions by protein cross-linking. Incubations were carried out as already described with the HLA-A3 probe. The nuclear protein amounts were either 6 (upper part) or 3 μg (lower part). The cross-linking reagent was added after allowing the DNA/protein(s) interactions to occur at 4°C for 20 min. The BS^3 concentrations were: 0, lanes 1 and 2; 0.18 mM (lane 3); 0.55 mM (lane 4); 1.66 mM (lane 5); 5 mM (lanes 6 and 7). Unlabeled probe (100 nM) was added for detecting non specific interactions (lanes 1 and 7).

detriment of B2. This was true whether B2 was elicited by means of high protein concentration or by adding OL1. At low protein concentration, where B2 was not detected in the absence of OL1, it may be speculated that BS^3 stabilized a latent B2 complex which had dissociated during the migration. If we assume that the B1 complex involves one DNA binding protein and B2 an additional one, the B3 complex, based on its lower mobility, should contain other component(s) whose DNA binding properties are weak (or not compatible with the gel shift assay) or a protein which interacts between the proteic parts of complexes in B1 and B2 without binding DNA directly. These interactions were stabilized by BS^3.

Complex stabilization by BS^3 enabled the obtention of the DNase I footprinting patterns in B1 and B2 (B3) once separated after gel retardation (not shown). The data obtained were similar to those presented in Fig. 1. Only the left part of the probes (Table I) was protected from DNase I action in B1. However the footprints obtained with B3 clearly revealed that the same molecule of DNA can be protected at two distinct locations even when the complexes are physically separated. There is no evidence for the involvement of a third DNA binding protein. It is tentatively concluded that B1 is part of B2 which had been stabilized as B3 upon BS^3 addition.

The H-DNA conformation hypothesis

The left parts of the 50-mer probes had a certain homology (see Table I). OL1 can compete for binding with this part of the probe. Since B1 complexes had similar mobilities whatever the probes, the same protein(s) were probably interacting with this part of the sequences. The same conclusions could a priori be drawn as far as B2 is concerned, this complex involving the right part (in Table I) of the 50-mer probes. However, the binding regions as determined from footprinting experiments obviously do not have any sequence homologies (Fig. 1 and Table I). We therefore questioned whether the recognition pattern was not dictated by a secondary structure more than by the sequence itself. It is indeed possible for the left part of the bottom strand of each probe (underlined part of the sequences in Table I) to form an internal triple helix (pairing rules in ref.35, see legend to Fig. 5) with the other end of the double-stranded probes (line above the sequences in Table I). Putative H-DNA conformations of the probes are shown in Fig. 5. Interestingly, the 3′ end of the fragment which is supposed to anneal to the right part of the probe, and the 5′ end of the latter correspond to the border of the footprint determined. The nucleotide sequences separating these two regions are long enough to allow the necessary DNA bending.

As shown for the HLA-A3 probe (Fig. 5, top), OL1 could anneal to the same region and generate an intermolecular triplex/single stranded H-DNA structure albeit with more

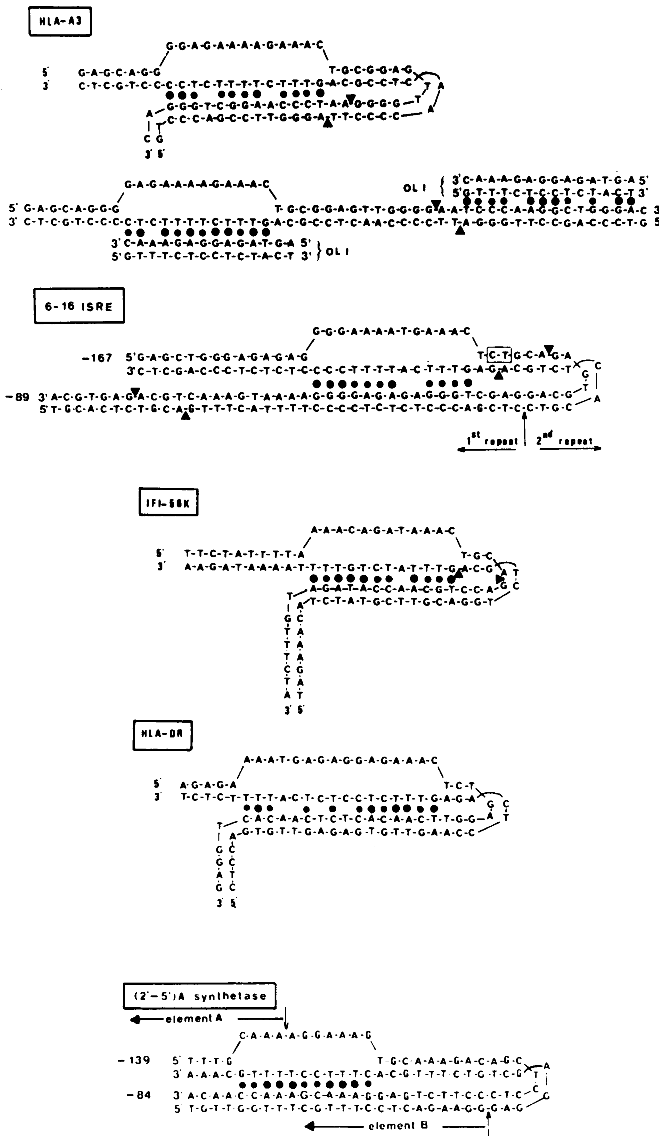

Fig. 5: Potentialities for the probes to form H-DNA structure. The pairing possibilities are those defined in ref.35. (T) can form an Hoogsteen link with an (AT) pair, (G) with (TA), (C) and to a lesser extent (T) with (GC) and (T), (C) or (G) with (CG). Large dots indicate more stable structures than those indicated by small dots. Cutting sites for restriction enzymes are indicated by black triangles (see Table I). The possibilities of hybridization of OL1 to each end of the probe are illustrated only for the HLA-A3 probe. In the case of the 6−16 gene, the two repeats are indicated; the sequence used as a probe in this study corresponds to the first repeat. The second repeat differs from the first one by the boxed CT bases. A potential H-DNA structure is also shown for human 2′,5′-oligoA synthetase; elements A and B are defined according to ref. 68.

mismatches. This might explain why OL1 was needed in greater quantities than the 50-mer probe to elicit B2 formation. Alternatively, such a structure could not be formed as easily since OL1 is not colinear with the probe.

Colinearity of pairing regions favored potential H-DNA containing complexes

A HinfI restriction site within HLA-A3 probe was used to physically dissociate the two interacting binding sites, each fragment including the entire zone protected from DNase I digestion. Each labeled fragment was tested for its ability to be recognized by proteins in the nuclear extract (note that when a fragment of the probe was labeled and tested for its binding, the other part of the 50-mer was present in equimolecular amount but left unlabeled)(Fig. 6). Only the entire probe was able to yield both B1 and B2 complexes with or without OL1. With the left part of the probe, only B1 was demonstrable, the right part of the probe being unable to induce any band shift. This suggests that the integrity of the molecule is necessary for promoting (or facilitating) B2 formation. OL1 alone allows B1 formation (see above). The addition of the right part of the probe (which cannot be recognized by nuclear extract proteins, but can anneal to OL1 (Fig. 5), promotes B2 formation without any evidence of the existence of B1 (since OL1 is not labeled). The prior formation of B1 thus seems a prerequisite for B2 to exist. In addition, the ability of OL1 to promote B2 formation when the left part of the probe is used, illustrates the symmetrical role of the regions involved in triple helix formation (see Fig. 5).

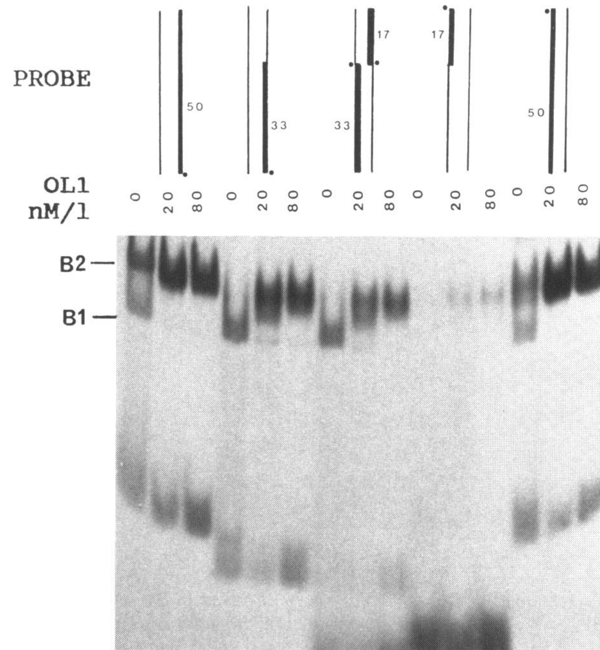

Fig. 6: Binding properties of probe fragments: effects of OL1 for generating triplex/single stranded complexes. HLA-A3 probe was cut with Hinf I (see Table I and Fig. 6) Various labeled fragments were generated (as indicated by the thick lines) depending on the experimental conditions (labeling before or after annealing; enzyme digestion before or after labeling). The length of the labeled fragment is indicated by the numbers along these lines, and the position of the label by a small dot. The full efficiency of restriction enzyme was verified. The ability of these fragments to recognize nuclear proteins (6 μg) was tested with 20 or 80 nM OL1 or in its absence.

It should be possible to determine which of the strands of OL1 participated in the B2 complex in an experiment analogous to that described in Fig.3. Neither strand of OL1 was able to be retarded nor to promote B2 formation. Whichever strand of OL1 was labeled, each of them was recovered in B2 when added to the nuclear extracts after annealing to its complementary strand (not shown). This is consistent with the potential of OL1 to hybridize to either portions (left or right part) of the 50-mer since the strand involved in the H-DNA structure would not be the same in either case (see Fig. 5).

Eight unrelated double-stranded oligonucleotides (16 to 23 mer) were randomly selected to demonstrate the specificity of OL1 (not shown). One of them promoted B2 formation: its sequence corresponded to the intergenic portion of the vesicular stomatitis

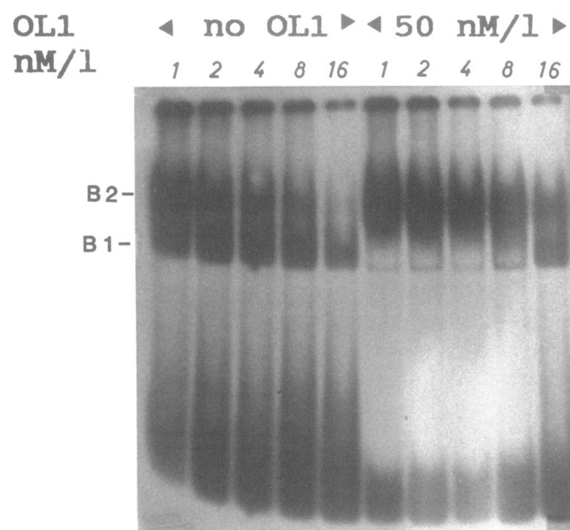

Fig. 7: Titration of DNA binding proteins involved in B1 and B2 complexes. The HLA-A3 probe concentration was 1 nM. In addition to the labeled probe, increasing amounts of unlabeled probe were added which yielded the final concentrations indicated at the top of the Fig. Nuclear extract protein concentrations was 6 μg.

virus (VSV in Table I). This sequence was potentially able to form a single/triple stranded structure.

Titration of B2 complex led to the reappearance of the B1 complex

The proteins involved in both complexes were titrated (Fig. 7): lowering the specific activity of the probe led to a faster loss of B2 than of B1 (left part, no OL1). Different patterns were observed when the complexes were formed in the presence of OL1 (Fig. 7, right part): higher concentrations of 50 mer were required to decrease significantly the amount of B2, concomittantly with the reappearance of B1. In fact increasing the labeled probe concentration titrated a preformed OL1/protein unlabeled complex (B1), therefore leading to B2 formation. When further increasing the probe concentration, the 50-mer competed with OL1 leading to : 1) labeled B1 complex (labeled 50 mer displacing unlabeled OL1 from B1) and 2) B2 complexes no longer involving both OL1 and the 50-mer probe but rather two molecules of the 50-mer. The H-DNA structure can in fact be generated within the same molecule of DNA or by allowing the combination of two molecules of the 50-mer probe. These observations are highly consistent with those of Fig. 3.

Potential secondary structure of the probes

It is difficult to ascertain which of the DNA or of the protein(s) favor the formation of such a multimolecular complex. We failed in our attempts to demonstrate the existence of such a triplex/single stranded structure in the naked DNA under the conditions of the binding reaction (pH 7.9). These conditions are obviously different from those generally used for demonstrating triple helix formation on naked DNA (38–46). These conditions were not necessarily compatible with the detection and stability of DNA-protein complexes. The formation of such structures was anyhow compatible with the loss of restriction enzyme site availability together with the occurence of complexes (not shown).

Enzymes such as DNase I and micrococcal nuclease reveal patterns which are suggestive of a structural organization of the probes themselves in the absence of added nuclear proteins (not shown). In the presence of Mn^{2+}, DNase I is known to cleave the DNA on both strands yielding blunt ended fragments, whereas with Mg^{2+}, the cleavages are randomly distributed on each strand (47). Thus with Mn^{2+}, a single cleavage may induce a complete loss of structure. In keeping with this, when low concentrations of DNase I (0.5 μg/ml, 1 min., 30°) were used in the presence of Mg^{2+}, the cleavage patterns did not yield a ladder as expected from statistical cleavage. A pattern common to all probes could not however be defined. When using Mn^{2+} instead of Mg^{2+} , for the same DNase I concentrations, the cleavage patterns were all the same and the probes were completely degraded.

Intramolecular H-DNA conformation needs ATP

DNA folding in H-DNA implies some constraints. B2 complex formation has been shown to depend upon ATP and IFN treatment (32). By adding OL1 to the nuclear extracts, the folding of the 50-mer DNA probe is no longer necessary since the two interacting species are not colinear. B2 can however be detected in the absence of ATP provided OL1 is present (not shown). This suggests that an energy requirement was no longer necessary. The fact that the appearance of B2 is still highly dependent on the protein concentration is compatible with a multifactorial interaction (not shown).

Extension of the results to other probes

The data presented above were obtained using the HLA-A3 probe. Similar qualitative data were obtained for the other probes listed in Table I and extended to other IFN inducible promoters (see conclusions). The convergence of the results and the compatibility of the proposed model with the data strongly support the H-DNA conformation hypothesis (Fig. 5).

CONCLUSIONS

The experiments presented in this paper were designed to further characterize the mechanisms enabling B2 formation. The primary observation was that titration of the nuclear factor(s) involved in the B1 complex facilitated the formation of the B2 complex, the proteins involved in the B1 complex being part of the B2 one. Similar observations were made for all the probes used despite significant differences in their sequences. A possible explanation is that such complexes result from secondary structure, non homologous nucleotide sequences being able to generate similar conformations.

The protein-independent topology and formation of DNA triple helices containing oligopurine-oligopyrimidine sequences is well established (48). Whether or not Hoogsteen base pairs occur in DNA is still an open question (49). In vitro, the formation of triple helix structure protects DNA from restriction endonucleases or methylases, inhibits Sp1 binding (50) and represses c-myc gene transcription (51). Evidence for an H-DNA structure involving both single and triple stranded DNA was derived from the sensitivity to nuclease S1 at promoter regions known to interact with transcriptional factors (52–55). Triplex formation has also been suggested to play a role in DNA bending and unwinding (56) or in affecting chromatin structure (57).

The proposed triplex/single stranded conformation model is in line with our experimental data although its direct occurrence could not be experimentally proven. The possibility of finding sequences in each of the probes obeying the triple helix pairing

rules as defined in Ref. 35 is in line with the proposed model. In the 2′,5′-oligoA synthetase case in particular, a perfectly matched triplex structure was observed. Moreover it has recently been shown that a 15-mer triplex, although significantly destabilized, could bear up to 3 mismatches (48). Whether the DNA interacting proteins or the DNA itself lead to the formation of such a complex is not clear; each of the partners most probably contributes for its own part to the information necessary to allow its building. The incubation conditions may also be of prime importance. For instance, the presence of a divalent cation changes the pairing rules (42,44,59)or conformational transition (59). Multiple hypotheses for DNA structure are possible due to the high degree of structural polymorphism shown by homopurine-homopyrimidine tracts (42,59). The patterns of DNase I and micrococcal nuclease sensitivities of the probes in the absence of nuclear proteins provide evidence that there is information superseding the informative content readable through the sequence.

The proposed model would explain why a complex such as B2 can easily be detected in a band shift assay, but difficult to characterize by footprinting experiments: a single break in the probes would probably be deleterious to a multimeric complex. The use of protein cross-linking reagent indeed allowed the detection of a DNA-protein complex (B3) of a lower mobility. This suggests that component(s) additional to B1 and B2 proteins interact(s) with the system, this component being not necessarily in direct interaction with the DNA (unpublished results and 60,61).

The hypothesis that a triple helix structure might be formed through the interaction of ribonucleoprotein(s) has been tested by analogy with observations described for c-myc gene (63,64). A pretreatment of the nuclear extracts or a treatment of the preformed complexes with RNase A did not modify the band shift pattern (not shown).

The 6-16 ISRE behaved similarily to the other probes in gel retardation experiments. The B2 complex should involve 2 molecules of the 6-16 ISRE to fit our model (Fig.6), whereas with the other probes an intramolecular folding of the DNA could be sufficient. It should be recalled that this sequence is almost fully duplicated in the natural gene (25). It also correlates very well with the 5′ deletion analysis of the 6-16 gene involving progressively the first and the second repeat and leading to a gradual loss of IFN inducibility (33). The further characterization of the interacting sequences with the 6-16 ISRE motif demonstrated the existence of two complexes, namely E and C1/C2 which bind to 'overlapping sites within the ISRE but in different ways. These data were used to design oligonucleotides which decreased the formation of the inducible complex without affecting the constitutive one'(33). If the formation of B2 (E) needs the prior formation of B1 (C1/C2), this explains the reported observation that no mutation within the 6-16 ISRE has been found which affects C1/C2 but not E. The conclusions obtained from the footprinting experiments by the authors were in accordance with the proposed model i.e. from the extensive overlap of their binding sites, the E and the C1/C2 complexes should not be able to bind to the same DNA molecule simultaneously (which does not exclude that the E complex may involve 2 molecules of DNA). Such a proposition will then exclude the hypothesis of gene regulation through mutual factor exclusion (26,65,66) for such promoters, but rather would reinforce the idea of synergistic cooperation.

The same analysis can be made concerning the human 2′,5′ oligoA synthetase promoter where the −136/−125 region (part of element A) could assume a H-DNA with the −97/−87 region (element B in ref.68) of the promoter.

ACKNOWLEDGEMENTS

The authors thank Institut Mérieux for its gift of $\alpha\beta$ IFN. This work has been funded by grants from CNRS and ARC to B.L. and from INSERM (CRE 881015) to C.R. We thank J.P. Leonetti and N. Mechti for stimulating discussions.

REFERENCES

1. Lengyel, P. (1982) Ann. Rev. Biochem. **51**, 251−282.
2. Pestka, S., Langer, J.A., Zoon, K.C. and Samuel, C.E. (1987) Ann. Rev. Biochem. **56**, 727−777.
3. Revel, M. and Chebath, J. (1986) Trends Biochem. Sci **11**, 166−170.
4. Zoon, K.C. and Arnheiter, H. (1984) Pharmac. Ther. **24**, 259−278.
5. Mogensen, K.E. and Bandu, M.T. (1983) Eur. J. Biochem. **134**, 355−364.
6. Anderson, P., Yip, Y.K. and Vilcek, J. (1982) J. Biol. Chem. **257**, 11301−11304.
7. Aguet, M., Dembic, Z. and Merlin, G. (1988) Cell **55**, 273−280.
8. Munro, S. and Maniatis, T. (1989) Proc. Natl. Acad. Sci. USA **86**, 9248−9252.
9. Hemmi, S., Peghini, P., Metzler, M., Merlin, G., Dembic, Z. and Aguet, M. (1989) Proc. Natl. Acad. Sci. USA **86**, 9901−9905.
10. Dale, T.C., Imam, A.A.M., Kerr, I.M. and Stark, G.R. (1989) Proc. Natl. Acad. Sci. USA **86**, 1203−1207.
11. Levy, D.E., Kessler, D.S., Pine, R. and Darnell,Jr.J.E. (1989) Genes Dev. **3**, 1362−1371.
12. Kusari, J. and Sen, G.C. (1987) Mol. Cell. Biol. **7**, 528−531.
13. Lew, D.J., Decker, T. and Darnell, Jr.J.E. (1989) Mol. Cell. Biol. **9**, 5404−5411.
14. Broxmeyer, H.E., Cooper, S., Rubin, B.Y. and Taylor, M W. (1985) J. Immunol. **135**, 2502−2506.
15. Czarniecki, C.W., Fennie, C.W., Powers, D.B. and Estell, D.A. (1984) J. Virol. **49**, 490−496.
16. Zerial, A., Hovanessian, A.G., Stefano, S., Huygen, K., Werner, G.H. and Falcoff, E. (1982) Antiviral Res. **2**, 227−239.
17. Levy, D.E., Lew, D.J., Decker, T., Kessler, D.S. and Darnell, Jr.E.J. (1990) EMBO J. **9**, 1105−1111.
18. Friedman, R.L. and Stark, G.R. (1985) Nature **314**, 637−639.
19. Wu, L.C., Morley, B.J. and Campbell, R.D. (1987) Cell **48**, 331−342.
20. Korber, B., Hood, L. and Stroynowski, I. (1987) Proc. Natl. Acad. Sci. USA **84**, 3380−3384.
21. Wathelet, M.G., Clauss, I.M., Nols, C.B., Content, J. and Huez, G.A. (1987) Eur. J. Biochem. **169**, 313−321.
22. Benech, P., Vigneron, M., Peretz, D., Revel, M. and Chebath, J. (1987) Mol. Cell. Biol. **7**, 4498−4504.
23. Hug, H., Costas, M., Staeheli, P., Aebi, M. and Weissmann, C. (1988) Mol. Cell. Biol. **8**, 3065−3079.
24. Reid, L.E., Brasnett, A.H., Gilbert, C.S., Porter, A.C.G., Gewert, D.R., Stark, G.R. and Kerr, I.M. (1989) Proc. Natl. Acad. Sci. USA **86**, 840−844.
25. Porter, A.C.G., Chernajovsky, Y., Dale, T.C., Gilbert, C.S., Stark, G.R. and Kerr, I.M. (1988) EMBO J. **7**, 85−92.
26. Levy, D.E., Kessler, D.S., Pine, R., Reich, N. and Darnell, J.E. (1988) Genes Dev. **2**, 383−393.
27. Kelly, J.M., Porter, A.C.G., Chernajovsky, Y., Gilbert, C.S., Stark, G.R. and Kerr, I.M. (1986) EMBO J. **5**, 1601−1606.
28. Samanta, H., Engel, D.A., Chao, H.M. and Thakurt, A. (1986) J. Biol. Chem. **261**, 11849−11858.
29. Rutherford, M.N., Hannigan, G.E. and Williams, B.R.G. (1988) EMBO J. **7**, 751−759.
30. Kessler, D.S., Levy, D.E. and Darnell, Jr.J.E. (1988) Proc. Natl. Acad. Sci. USA **85**, 8521−8525.
31. Roy, C. and Lebleu, B. (1989) Biochem. Biophys. Res. Commun. **163**, 370−377.
32. Roy, C. and Lebleu, B. (1990) Nucl. Acids Res. **18**, 2125−2131.
33. Dale, T.C., Rosen, J.M., Guille, M.J., Lewin, A.R., Porter, A.G.C., Kerr, I.M. and Stark, G.R. (1989) EMBO J. **8**, 831 -839.
34. Cohen, B., Peretz, D., Vaiman, D., Benech, P. and Chebath, J. (1988) EMBO J. **8**, 1411−1419.
35. Griffin, L.C. and Dervan, P.B. (1989) Science **245**, 967−971.
36. Dignam, J.D., Lebovitz, R.M. and Roeder, R.G. (1983) Nucl. Acids Res. **11**, 1475−1489.

37. Bradford, M.M. (1976) Anal. Biochem. **72**, 248–254.
38. Francois, J.C., Saison-Behmoaras, T. and Hélène, C. (1988) Nucl. Acids Res. **16**, 11431–11440.
39. Moser, H.E. and Dervan, P.B. (1987) Science **238**, 645–650.
40. de Los Santos, C., Rosen, M. and Patel, D. (1989) Biochemistry **28**, 7282–7289.
41. Lyamichev, V.I., Frank-Kamenetski, M. and Sogfer, V.N. (1990) Nature **344**, 568–570.
42. Shimizu, M., Hanvey, J.C. and Wells, R.D. (1990) Biochemistry **29**, 4704–4713.
43. Hanvey, J.C., Shimizu, M. and Wells, R.D. (1988) Proc. Natl. Acad. Sci. USA **85**, 6292–6296.
44. Kohwi, Y. and Kohwi-Shigematsu, J.(1988) Proc. Natl. Acad. Sci. USA **85**, 3781–3785.
45. Francois, J.C., Saison-Behmoaras, T., Barbier, C., Chassignol, M., Thuong, N.T. and Hélène, C. (1989) Proc. Natl. Acad. Sci. USA **86**, 9702–9706.
46. Sklenar, V. and Feigon, J. (1990) Nature **345**, 836–838.
47. Melgar, E. and Goldthwaite, D.A. (1968) J. Biol. Chem. **243**, 4409–4416.
48. Htun, H. and Dahlberg, J.E.(1989) Science **243**, 1571–1576.
49. Portugal, J.(1989) Trends Biochem. Sci.**14**, 127–130.
50. Maher III, L.J., Wold, B. and Dervan, P.B. (1989) Science **245**, 725–730.
51. Cooney, M., Czernuszewicz, G., Postel, E. H., Flint, S.J. and Hogan, M.E. (1988) Science **241**, 456–459.
52. Johnson, A.C., Jinno, Y. and Merlino, G.T. (1988) Mol. Cell. Biol. **8**, 4174–4184.
53. Postel, E.H., Mango, S.E. and Flint, S.J. (1989) Mol. Cell. Biol. **9**, 5123–5133.
54. Hoffman, E.K., Stephen, S.P., Murphy, M. and George, D.L. (1990) Proc. Natl. Acad. Sci. USA 87, 2705–2709.
55. Boles, T.C. and Hogan, M.E. (1987) Biochemistry, **26**, 367–376.
56. Caddle, M.S., Lussier,R.H. and Heintz, N.H. (1990), J. Mol. Biol. **211**, 19–33.
57. Gilmour, D.S., Thomas, G.H. and Elgin, S.C.R. (1989) Science **245**, 1487–1490.
58. Shea, R.G., Ng, P. and Bischofberger, N. (1990) Nucl. Acids Res. **18**, 4859–4866.
59. Bernués, J., Beltran, R., Casasnovas, J.M. and Azorin, F. (1989) EMBO J. **8**, 2087–2094.
60. Fu, X.Y., Kessler, D.S., Veals, S.A., Levy, D.E. and Darnell, J.E.Jr. (1990) Proc. Natl. Acad. Sci. USA **87**, 8555–8559.
61. Kessler, D.S., Veals, S.A., Fu, X.Y. and Levy, D.E. (1990) Genes Dev. **4**, 1753–1765.
62. Davis, T.L., Firulli, A.B. and Kinniburg, A.J. (1989) Proc. Natl. Acad. Sci USA **86**, 9682–9686.
63. Kinniburg, A.J. (1989) Nucl. Acids Res. **17**, 7771–7778.
64. Lichtsteiner, S., Wuarin, J. and Schibler, U. (1987) Cell **51**, 963–973.
65. Goodbourn, S., Burstein, H. and Maniatis, T. (1986) Cell **45**, 601–610.
66. Dorn, A., Bollekens, J., Staub, A., Benoist, C. and Mathis, D. (1987) Cell **50**, 863–872.
67. Santaro, C., Mermod, N., Andrews, P.C. and Tjian, R. (1988) Nature **334**, 218–224.
68. Cohen, B., Vaiman, D. and Chebath, J. (1989) Nucl. Acids Res **17**., 1679–1695.