Nucleic Acids Research, 1994, Vol. 22, No. 15 3033–3037

In vivo footprinting of the IRF-1 promoter: inducible occupation of a GAS element next to a persistent structural alteration of the DNA

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Received May 10, 1994; Revised and Accepted June 26, 1994

ABSTRACT

GAS (gamma activated sequence) and GAS-like elements are found in a rapidly growing number of genes. Data from EMSA (electromobility shift assay) and transient transfection assays using heterologous promoter systems do not necessarily reflect transcriptional involvement and protein occupation of a binding site *in vivo*. This has been shown recently by *in vivo* footprinting of the NF-κB site at −40 in the interferon regulatory factor-1 (IRF-1) promoter. Here we show by *in vivo* footprinting using dimethylsulfate (DMS) that the GAS of the IRF-1 promoter, which also contains an overlapping putative NF-κB site, is occupied upon treatment with γ-interferon (IFNγ) but not with phorbol 12-myristate 13-acetate (PMA). Irrespective of induction, we detect a very strong DMS hypersensitivity at a guanosine just adjacent to GAS and a less persistent minor DMS hypersensitivity at a central cytosine. Our data confirm the crucial role of GAS in transcriptional activation by IFNγ and are consistent with induced binding of p91 to GAS. In addition, our data suggest a major conformational distortion of the DNA at the GAS element of the IRF-1 promoter and that this GAS element is not involved in transcriptional activation by PMA.

INTRODUCTION

Interferon regulatory factor-1 (IRF-1) was originally described as a nuclear factor specifically binding to *cis*-regulatory elements in the β-interferon (IFNβ) enhancer (1, 2). IRF-1 is identical with interferon-stimulated gene factor 2 (ISGF2) (3) described previously as a DNA-binding protein synthesized in response to interferons (IFNs) (4, 5). A great variety of agents have been shown to enhance IRF-1 gene transcription, including double-stranded RNA, IFNs, several pro-inflammatory cytokines, viruses, and phorbol esters (2, 6). IRF-1 was shown to play a critical role in the regulation of IFNβ at least in certain cells (7); it can affect the expression levels of IFNβ and IFN-inducible gene expression depending on the differentiation state of the cells (for discussion see ref. 8). The importance of IRF-1 is emphasized by the findings that it may play a role in the inhibition of cell growth (9, 10) and manifests anti-oncogenic properties in NIH 3T3 cells (11, 12). Moreover, it has been shown recently that IRF-1 is required for the induction of NO synthase in macrophages (13).

IFNs elicit antiviral and regulatory activities through the induction of IRF-1 and a variety of other genes (14–16). The IFN signal transduction pathways are among the best studied pathways known to date. The IFN signalling components comprise two novel protein families: the JAK family of non-receptor protein tyrosine kinases and the STAT family of latent cytoplasmic signal transducers and transcription factors (17–19, reviewed in 20–22). In response to IFNγ, a member of the STAT family, p91, is activated by tyrosine phosphorylation (23). The absolute requirement for p91 in the IFNγ response pathway has been demonstrated by genetic complementation of an IFN-unresponsive mutant cell line that does not express p91 (18). Activated p91 binds to GAS (gamma activated sequence), a *cis*-element first identified in the promoter of the guanylate-binding protein (GBP) gene (24). Subsequently, GAS and GAS-like sequences have been described in many genes inducible by IFNs and other cytokines (see 25 for examples). The signal transduction pathways of a variety of stimuli converge in the activation of p91 and p91-related polypeptides binding to GAS or GAS-like elements in a variety of promoters (25–33.) The GAS consensus sequence has been defined as 5′-TTNCNNNAA-3′ (reviewed in 21). It is therefore an important issue to investigate the affinities of the different GAS elements to p91 *in vitro* and *in vivo* to shed some light on the fine tuning of p91 action.

By sequence inspection several putative *cis*-regulatory motifs have been identified in the IRF-1 promoter (34, 35). A GAS element at position −120 has been characterized by transient transfection analysis and *in vitro* binding assays of p91 (25, 26, 35). This GAS element is located within an inverted repeat and overlaps with a hypothetical NF-κB site. NF-κB DNA-binding acitivity is known to be inducible by a variety of agents, including phorbol 12-myristate 13-acetate (PMA) (36). Very recently it has been demonstrated that an evolutionarily conserved, high-affinity

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NF-κB site (position −47 to −38) in the IRF-1 promoter is transcriptionally inactive *in vivo* (37).

In this study we investigated the IRF-1 promoter (position −150 to −20) by *in vivo* dimethylsulfate (DMS) footprinting upon induction with PMA or IFNγ. IFNγ induces protein occupation at the GAS element around position −120. p91 is the most likely protein to bind here, consistent with the aforementioned *in vitro* investigations (26). However, PMA does not alter the footprint pattern *in vivo* in accordance with previous *in vitro* findings (35). In addition, we detected irrespective of induction a strong DMS hypersensitivity at a guanosine and a minor one at a cytosine at the GAS element. This is indicative for a possible implication of DNA secondary structure in IRF-1 gene expression.

MATERIALS AND METHODS

Genomic sequencing and *in vivo* footprinting

Base-specific modification *in vitro* and piperidine cleavage was performed as described (38). The cleaved DNA was subjected to LMPCR (ligation-mediated PCR) as described below.

For *in vivo* footprinting with DMS, L929 cells were grown to subconfluency in RPMI supplemented with 10% fetal calf serum (FCS) and induced with 50 ng/ml PMA or with 1000 U/ml human IFNγ for 1 h. Cells were harvested and incubated in RPMI/10%FCS containing 0.2% DMS (Merck) at 3×10^7 cells/ml. The methylation reaction was carried out at room temperature for 2 min and was stopped by the addition of 40 vol. cold phosphate-buffer with 2% β-mercaptoethanol and the subsequent removal of the medium by centrifugation. Cells remain viable after this treatment as determined by trypan blue exclusion. The DNA was extracted by a standard protocol and cleaved at modified residues with piperidine. To visualize the DNA sequence the LMPCR-method was used essentially as described (39) with the exception that the polymerisation buffer consisted of 40 mM NaCl, 10 mM Tris−HCl, pH 8.9, 0.01% gelatine, 5 mM $MgSO_4$. For the extension step with the radioactive primer E four PCR cycles were performed. Sequencing gels and autoradiography were as described in (40).

The primers C, D, and E were used to analyse the sense-strand:

Primer C: 5′-GGTTCGGCTTAGACTGTGAAAGCACG-3′,
Primer D: 5′-CTGTGAAAGCACGTCCTACCTCGACG-3′,
Primer E: 5′-CTACCTCGACGAAGGAGTGGTGCGC-3′.

For analysis of the antisense-strand the following primers were used:

Primer C′: 5′-CCCCGGGGCGGTGGCGCGGGC-3′,
Primer D′: 5′-GGCGCGGGCCCGAGGGGGTGG-3′,
Primer E′: 5′-AGGGGGTGGGGAGCACAGCTGCC-3′.

RNA analysis

RNA analysis was performed as described (18). The hybridisation probe for IRF-1 RNA was an *ApaI*/*Hin*dIII fragment from plasmid pIRF299-wt (2), spanning the mRNA sequence from +1 to +225. The hybridisation probe for detection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was a DNA covering the sequence from +539 to +1036 of the human cDNA (41). This PCR product was obtained by using the oligonucleotides 5′-CCACCACCCTGTTGCTGTAGCC-3′ and 5′-TGGCCAAGGTCATCCTGACAACTT-3′ and was a kind gift of E.Kaiser.

RESULTS

PMA and IFNγ induce IRF-1 gene transcription

Northern analysis was performed to test the transcriptional activity of the IRF-1 promoter in L929 cells (Fig. 1). The IRF-1 promoter shows a low basal transcriptional activity in uninduced cells (Fig. 1, lane 1) and is inducible with IFNγ (Fig. 1, lane 2) or PMA (Fig. 1, lane 3). These data are consistent with observations of other investigators (6, 17). Hence, using this cell system for the *in vivo* footprinting analysis, we compare *in vivo* protein/DNA interactions of the IRF-1 promoter at low basal activity with those at enhanced activity by PMA or IFNγ.

Persistent features of *in vivo* protein occupation of the IRF-1 promoter

The *in vivo* DNA methylation patterns of uninduced (Fig. 2, lanes 1 and 5; Fig. 3, lane 5) as well as PMA- (Fig. 2, lanes 2 and 6) or IFNγ- (Fig. 3, lanes 3 and 6) induced L929 cells on both strands are clearly different from those obtained with naked DNA methylated *in vitro* with DMS (Fig. 2, lanes 3, 4 and 7; Fig. 3, lanes 2 and 4; compare also summary in Fig. 4). This indicates that several proteins stably occupy the IRF-1 promoter *in vivo*. The pattern of protein occupation shows constitutive features in that it remains stable after induction with PMA or IFNγ. The constitutively occupied sites are well characterized *cis*-acting elements, i.e. the CCAAT box and a GC-rich sequence which is a potential Sp1 binding site (42). The occupation of these transcriptional activator binding sites prior to induction parallels the low, but detectable activity of the uninduced IRF-1 promoter (Fig. 1, lane 1).

An unusually strong DMS hypersensitivity reveals a permanently distorted DNA structure *in vivo* at the GAS region

A striking DMS hypersensitivity *in vivo* was found at a guanosine located at position −131 in the upper strand (Fig. 2, lanes 5 and 6; Fig. 3, lanes 5 and 6; summary in Fig. 4). This unusually strong hypersensitivity is indicative of an enhanced exposure of this particular residue to DMS, and hence a strong indication of a distortion in DNA secondary structure. A second minor DMS hypersensitivity occasionally observed at a cytosine at position −122 (Fig. 2, lanes 5 and 6) further supports this: the C-methylation presumably occurs at position N-3, which is DMS-accessible only in structurally distorted DNA (43, and refs therein). Since this susceptibility of the cytosine residue is also

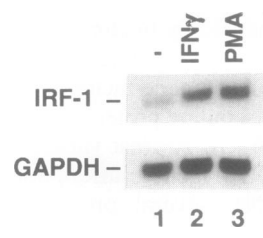

Figure 1. RNA analysis of uninduced, PMA-induced and IFNγ-induced L929 cells. Total cytoplasmic RNAs from uninduced (lane 1), PMA-induced (lane 2) and IFNγ-induced (lane 3) L929 cells were analysed by gel electrophoresis, blotting and filter hybridisation with probes for IRF-1 or GAPDH as described in Materials and Methods.

observed to some extent *in vitro* (Fig. 2, lane 4), it appears that the DNA region containing the DMS hypersensitivities is generally prone to structural alteration.

PMA induction of IRF-1 transcription does not influence protein/DNA interactions

PMA treatment of cells does not alter the footprinting pattern of the IRF-1 promoter on both strands (compare lanes 1 with 2, and 5 with 6 in Fig. 2). No footprint is detectable around position −120 (denoted by 'IR, NF-$\varkappa$B?' in Figs 2 and 4), which has been suggested to constitute an NF-$\varkappa$B site (35). This argues against an involvement of NF-$\varkappa$B by binding to this site *in vivo* and is consistent with results obtained *in vitro* (35). We conclude from the identical footprinting patterns of uninduced and PMA-induced L929 cells that the signal transduction pathway of PMA does not involve protein−DNA interactions established *de novo* at the investigated promoter region.

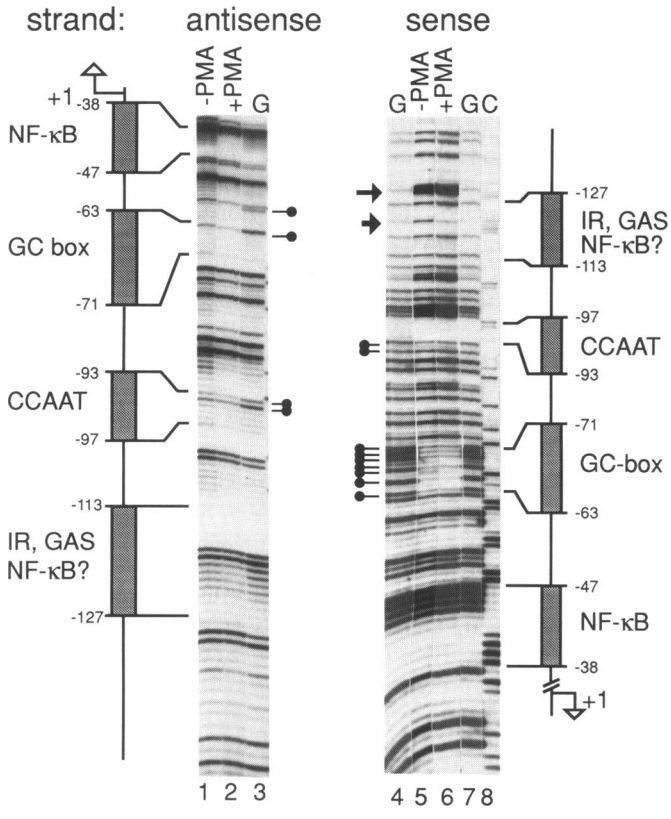

Figure 2. *In vivo* footprint analysis of L929 cells; induction with PMA. Genomic DNA from L929 cells was modified *in vitro* (lanes 3, 4, 7, and 8; G indicates guanosines methylated with DMS; C indicates cytidines modified with hydrazine) or *in vivo* (lanes 1, 2, 5, and 6; modification with DMS was performed in the absence or presence of PMA, as indicated) and specific regions were detected with LMPCR as described in Materials and Methods. Amplification products were analysed by sequencing gel electrophoresis and autoradiography. The localization of the NF-$\varkappa$B, GC, CCAAT, and inverted repeat/GAS sites (grey boxes) is indicated by numbering the nucleotides upstream from the transcription initiation point (+1). Lines with black dots denote decreases, arrows denote increases in intensity of *in vivo* (± PMA induction) versus *in vitro* modified DNA. The length of the symbols represents the approximate degree of protection/hypersensitivity.

Induction of IRF-1 gene transcription by IFNγ leads to stable occupation of GAS

The inverted repeat around position −120 in the IRF-1 promoter encompasses a GAS element which has been suggested to mediate activation of IRF-1 gene transcription by IFNγ (25, 26, 35). In agreement with these results, induction with IFNγ leads to clear footprints *in vivo* at the GAS on both strands (Fig. 3, lanes 3 and 6). GAS occupation is apparent as guanosine protections underlined in the sequence

5′-TTCCCCGAA-3′
3′-AAGGGGCTT-5′

which has been characterized as the core binding sequence of protein p91 (25, 26). Therefore, it is reasonable to assume that p91 causes the observed IFNγ inducible *in vivo* footprinting pattern. Furthermore, the inducible protein occupation at GAS occurs next to a region of a preformed distortion of the DNA secondary structure (see above), without any further changes.

DISCUSSION

The commonly used transfection of recombinant plasmids containing normal and mutant sites of DNA−protein interactions

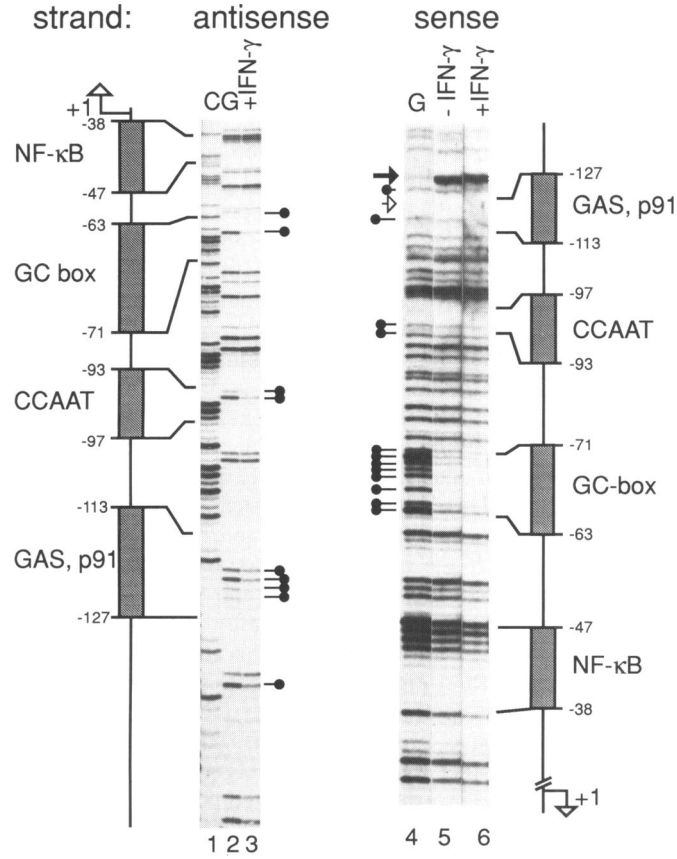

Figure 3. *In vivo* footprint analysis of L929 cells; induction with IFNγ. Description and symbols are as in Fig. 2. Lines with black dots at the GAS denote decreases in intensity only in IFNγ-induced cells. The open arrow marks the position of the DMS-reactive cytosine (cf. Fig. 2), which is scarcely visible here. Lanes 1, 2 and 4 represent genomic sequencing, lanes 3, 5, and 6 *in vivo* footprinting of the respective DNA strand.

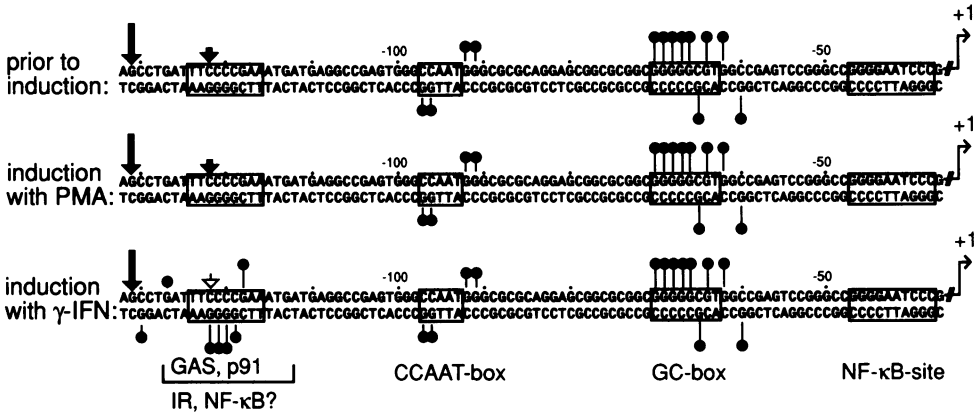

Figure 4. Summary and comparison of the *in vivo* footprinting data at the IRF-1 promoter in uninduced, PMA-induced and IFNγ-induced L929 cells. Symbols are as in Figs. 2 and 3.

is a valuable tool for studying canditate regulatory sites and their binding proteins. However, these assays cannot take into account the effects of copy numbers, chromosomal context, and DNA topology. To monitor DNA–protein interactions and DNA structure during gene activation in the original chromosomal context, we analysed protein binding to the mouse IRF-1 promoter in uninduced and PMA- or IFNγ-induced L929 cells by DMS footprinting *in vivo*. Constitutive protein occupation was detected on both DNA strands of the CCAAT box and the GC box (see Fig. 4). The latter is most likely occupied by transcription factor Sp1 (42). Protein binding to these important *cis*-regulatory elements prior to induction correlates with a low basal transcriptional activity of the IRF-1 promoter as described here (Fig. 1) and elsewhere (6). It has been shown recently that the NF-κB binding site from position −38 to −47 in the IRF-1 promoter binds NF-κB with high affinity *in vitro* but is transcriptionally inactive *in vivo* upon stimulation with PMA (37). Other binding sites for NF-κB may, therefore, be responsible for mediating the transcriptional stimulation of the IRF-1 promoter. Since the inverted repeat around position −120 contains a putative NF-κB site (35) we monitored this site after PMA stimulation by *in vivo* footprinting. PMA induction does not induce protein occupation at this site and does not alter the footprint pattern. Therefore, PMA-mediated induction must occur via protein–protein interactions and/or protein–DNA interactions outside the investigated promoter region.

The protein occupations of the GC and CCAAT boxes are constitutive, i. e. they are not altered after induction of IRF-1 expression with either PMA or IFNγ. This and the RNA analysis indicate that the IRF-1 promoter belongs to the immediate early genes requiring a preformed protein–DNA architecture to become rapidly active. This has been detected by *in vivo* footprint analyses also at other rapidly inducible genes (44–46).

The DNA at the inverted repeat around position −120 of the IRF-1 promoter shows constitutive structural alterations *in vivo* as indicated by the presence of a strong DMS hyperreactivity of the guanosine at position −131 in the upper strand and a minor variant DMS hyperreactivity at a cytosine at position −122. This distortion in DNA secondary structure associated with a GAS element is described here for the first time. What could be the cause of this structural alteration? In principle the short inverted repeat might form a short-lived cruciform stabilized by proteins and/or torsional strain of the DNA. The DMS hyperreactive cytosine at position −122 is the first nucleoside next to a loop in the hypothetical cruciform structure, which could explain the enhanced DMS reactivity (cf. ref. 47). A more probable structure is a DNA bend or kink induced and/or stabilized by protein binding, for example, the formation of a nucleosome (48, 49). The organization of the IRF-1 promoter within a nucleosome *per se* does not preclude the binding of other proteins at the positive *cis*-regulatory elements CCAAT box and GC box; the functional interplay of a nucleosome and non-histone proteins has been inferred also from studies of other promoters (e.g. 50, 51). In this context, it is interesting to note, that in the case of another prominent member of the IFN network, namely IFNβ, HMG I(Y) binding of the NF-κB binding site (PRDII) and of the ATF-2 binding site (PRDIV) occurs and is required for full inducibility in response to virus infection (52, 53). Nucleosomes (48), as well as HMG proteins (54) are known to bind to the minor groove and to bend DNA. Binding in the minor groove would not have been detected by DMS footprinting which resolves guanosine contacts in the major groove. Accordingly, induction with IFNγ leaves the DMS hyperreactivity at guanosine −131 unchanged but induces protein occupation at the major groove nearby.

There is growing evidence that the inverted repeat at position −120 encompassing a GAS is involved in mediating induction of transcription by IFNγ (25, 26, 35). In this study we show an IFNγ-inducible protein occupation *in vivo* exactly at the GAS on both DNA strands. Since it has been shown recently that tyrosine phosphorylated p91 from IFNγ-treated cells binds to the GAS of the IRF-1 promoter *in vitro* (25, 26), we suggest that binding of phosphorylated p91 causes the IFNγ-inducible DMS protection *in vivo*. Footprints of a GAS have been shown to occur *in vivo* also at the promoter of GBP (55). However, the *in vivo* studies of the GAS occupation in the GBP promoter are difficult to interpret, because only one out of two guanosines in the GAS is protected and because of the simultaneous occupation of an overlapping interferon-stimulating response element.

The different footprint patterns of the IRF-1 promoter after PMA or IFNγ induction reflect the different signal transduction pathways which in this case converge not before the level of protein–DNA interaction to stimulate gene transcription. On the other hand, various other agents converge at the level of p91 activation by phosphorylation (26), and p91 itself may be

dispersed at many potential binding sites (25–32). Our results clearly show inducible occupation of the GAS element adjacent to a persistent deformation of the DNA structure in the IRF-1 promoter and thereby add to the important issue of elucidating the regulation and action of p91. Further examination of different activators and targets of p91 *in vitro* and *in vivo* is required to complete the understanding of the fine tuning of p91 function.

ACKNOWLEDGEMENTS

We thank L.Zeitlmann for preparing the IRF-1 and E.Kaiser for kindly providing the GAPDH hybridisation probe, H.Ibelgaufts for critical reading of the manuscript and Prof. E.-L.Winnacker for continuous support. This work was supported by the Deutsche Forschungsgemeinschft (Zo 59/2-1).

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