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Constitutive in vitro binding of nuclear proteins to the 5'-flanking region of 6-16, a human gene inducible by α, β-interferons

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Proteins in nuclear extracts of HeLa cells that constitutively bound in vitro to three regions upstream of the interferon-inducible gene 6-16 were separated partially by chromatography on DEAE-Sepharose Region one, a CCAAT box in the non-coding strand at position −63 to −67, was protected from DNase digestion by the bound protein(s) and was required for transcription in vitro Region two, a tandem duplication sequence at position −89 to −168 contains two copies of a sequence essential for strong induction of the 6-16 gene by interferon in vivo Region three, a palindromic sequence at position −449 to −465, not necessary for induction of 6-16 by interferon, was also protected from DNase digestion by nuclear protein(s) Templates with or without regions of two and three were transcribed equally well in extracts from interferon-treated or untreated cells

Transcription, in vitro, Interferon regulation, Transcription factor

1. INTRODUCTION

Detailed studies of transcriptional regulation by interferons have been made possible by cloning cDNAs corresponding to interferon-inducible mRNAs and the genes themselves ([1-8]; see [9] for a recent review). Some of these genes are induced by any of the interferons, whereas others are induced predominantly by type I (α,β) or type II (γ) interferon. An example of the latter is the human gene 6-16, which is strongly induced by type I interferon in a variety of different cells [4,10, 11]. The 6-16 gene, which encodes a small protein of unknown function, is essentially silent in untreated cells. The mRNA accumulates transiently in the nucleus and steadily in the cytoplasm of interferon-treated cells [10-12], usually as a primary response insensitive to inhibitors of protein synthesis such as cycloheximide [10].

We have defined regions required in vivo for induction of 6-16 and for its basal expression by deleting DNA sequences upstream of the gene [1]. This work has shown that a directly duplicated sequence at position −89 to −168 is responsible for most of the regulatory effect of interferon on the 6-16 gene. The same region can confer inducibility by interferon when placed upstream of a heterologous marker gene. Tandemly reiterated regulatory elements are not uncommon and have been reported previously for heat shock, β1-interferon, metallothionein and glucocorticoid-regulated genes (reviewed in [13]).

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Transcription and protein-DNA binding experiments have shown that subelements of inducible enhancers can interact specifically with nuclear proteins in vitro. Sometimes the binding activities of these proteins are increased in response to the inducer, and sometimes they are not. There is an increase in the activity of proteins that bind to heat shock elements in *Drosophila* and HeLa cells in response to increased temperature [14,15]. The proto-oncogene *c*-fos is highly induced by a variety of stimuli, including growth factors, cyclic AMP, calcium ionophores and phorbol esters. In this case, proteins that bind to the enhancer element are found in nuclear extracts from both induced and uninduced HeLa cells [16]. In contrast, extracts of A431 cells contain an activity which binds to the *c*-fos enhancer only when the cells have been treated with epidermal growth factor [17]. The enhancer elements of the interferon-regulated mouse H-2k and β2-microglobulin genes bind to nuclear proteins in vitro whether the cells have been treated with or without interferon [18]. On the other hand, Levy et al. [19] reported that they can detect interferon-modulated protein binding to the enhancer element of the ISG-54 gene in vitro. Similarly Porter et al. [1] showed interferon-specific band shifts with an oligonucleotide corresponding to the 3' unit of the direct repeat of the 6-16 promoter, and Rutherford et al. [20] with a promoter fragment of the 2'-5' oligo A synthetase gene. To further our understanding of how specific protein-DNA interactions may regulate expression of the 6-16 gene, we have studied protein binding to three regions upstream of the mRNA start site. In no case was a dependence on interferon treatment observed when the whole promoter region of 6-16 was

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used. We relate these findings to in vitro transcription assays and to the in vivo results of Porter et al. [1].

2. MATERIALS AND METHODS

2.1 *Plasmids for in vitro transcription assays*

The restriction sites used are shown in fig 1. The 3.2 kb *Xho*I-*Asp*718 (*Kpn*I) fragment from cosmid 10.3 [11], containing 2300 nucleotides upstream and 900 nucleotides downstream of the start point of transcription and including the first exon of the 6-16 gene, was cloned between the *Sal*I and *Asp*718 sites of the polylinker region of plasmid pUC18 [21]. The deleted plasmid *ΔBgl*II was obtained by subcloning the 1.5 kb *Bgl*II-*Asp*718 fragment between the *Bam*HI and *Asp*718 sites of pUC18. This recombinant plasmid was then digested with *Sph*I, which cuts in the upstream polylinker region and 207 nucleotides downstream of the *Bgl*II site in the 6-16 promoter, and then religated to yield *ΔSph*I *ΔPst*I was obtained in a similar manner with *Pst*I, which cuts in the upstream polylinker and 508 nucleotides downstream of the *Bgl*II site.

2.2 *Plasmids for gel retardation assays*

*ΔBgl*II was cleaved with *Hpa*II and the protruding ends were filled in by use of the Klenow fragment of DNA polymerase. The 0.9 kb *Hpa*II fragment was isolated, cut in the polylinker with *Hin*dIII and the resulting fragment, containing the sequences from −5 to −603, was cloned between the *Sma*I and *Hin*dIII sites of pUC18 to yield *ΔBgl*II-*Hpa*II. For gel retardation assays, either the *Hin*dIII or the *Asp*718 site was labelled with ^{32}P and the small fragment was cut out with *Bcl*I, which cleaves 293 nucleotides downstream of the *Bgl*II site.

2.3 *In vitro transcription assays*

Extracts were prepared as described by Dignam et al. [22] and the reactions were carried out as described by these authors but using 3 mM $MgCl_2$ and 1 mM spermidine. Each plasmid template was cut with *Eco*RI before use. Transcripts were synthesized in the presence of unlabelled ribonucleotides and the products were hybridized with 15 pmol of the ^{32}P-labelled primer 5′-CTTGGAGGAGAGAAGAG-AAG-3′, which corresponds to the most downstream 20 nucleotides of the first exon of 6-16. The primer was then extended with reverse transcriptase [11], the cDNA was separated in a 20% polyacrylamide gel and visualized by autoradiography at −70°C overnight with an intensifier screen, using Kodak XAR5 film.

2.4 *Gel retardation assays*

When unfractionated nuclear extracts were used, an amount containing 1 μg of protein was incubated at room temperature in 20 μl with 1 μg of poly(dI dC), 5000 cpm of labelled fragment (approximately 1 ng of DNA) in 10% glycerol, 20 mM Hepes buffer, pH 7.9, 75 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethanesulfonylfluoride and 5 mM $MgCl_2$ for 30 min. For the amounts of protein used in assays of fractionated extracts, see the legend to fig 4. The reaction mixtures were loaded onto 6% acrylamide-bis acrylamide gels (30.0.8) in buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.3). The gels were pre-run for 2 h at 30 mA and the samples were run at 20 mA for 2–4 h, depending on the size of the labelled fragment. The gels were dried and autoradiographed as described above.

2.5 *DNase footprinting*

End-labelled DNA was allowed to bind with 10 μg of protein from the 0.225 M KCl fraction of the DEAE-Sepharose column in 20 μl, as in the retardation assays, and 2 μl of DNase I [Worthington, 50 μg/ml stock solution] were added. The reaction mixtures were incubated for 1 min at room temperature and 2 μl of 0.25 M EDTA were added to stop the reaction. The complexes produced in five binding reactions were separated from free fragments by gel electrophoresis. The wet gel was autoradiographed and the retarded complexes were isolated by crushing the gel through a syringe into 25 ml of proteinase K (0.2 mg/ml) in 40 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 20 mM EDTA and 0.5% SDS. After shaking overnight at 37°C, the mixture was passed through siliconized glass wool to remove the acrylamide particles and extracted twice with phenol-chloroform (1·1). The DNA was concentrated by binding to a column of DEAE 52-cellulose (0.3 ml of bed volume) and eluted with 1.5 M NaCl, 20 mM Tris-HCl, pH 8.0, 2 mM EDTA. After precipitation with ethanol in the presence of 10 μg of carrier tRNA, the DNA was resuspended in formamide dye [11], denatured by boiling and separated by electrophoresis in a sequencing gel.

2.6 *Plasmids and construction of the expression vectors*

For subcloning purposes, we used pUC19 [21] and for the construction of an interferon-inducible promoter, we cloned two oligonucleotides representing the two units of the direct repeat of the 6-16 promoter in the polylinker region of the reporter plasmid pBLCAT8+ [23]. The cloning of the direct repeat sequence was done by self ligation of the T4 kinase-treated oligonucleotides corresponding to the 5′ and 3′ units of the direct repeat (fig 3), followed by restriction with *Taq*I which cuts the circularized self-ligated oligonucleotide producing a complete unit of the direct repeat sequence of 6-16. This fragment was cloned into the *Acc*I site of pUC19. Positive clones were detected by hybridization to [^{32}P]-end-labelled oligonucleotides. The orientation of the inserts was determined by growing the *Escherichia coli* DH5α bacteria in plates with IPTG and X-gal. The tandem repeat in the opposite orientation with respect to the β-galactosidase gene produced blue colonies because it continued the open reading frame (ORF) of the enzyme. Therefore, we used a clone containing the opposite orientation of the direct repeat to isolate the *Bam*HI-*Hin*dIII insert and cloned it 5′ to the CCAAT box of the tk promoter in the plasmid pBLCAT8+. The plasmid was called D.R pBLCAT8+ and had the tandem repeat in the same orientation as in the 6-16 native promoter.

In order to study the function of the CCAAT box, we removed it from the D.R pBLCAT8+ by isolating the *Xba*I-*Bgl*II fragment and cutting it again with *Hha*I which cuts at position −48, removing the CCAAT box. We then cloned back the *Hha*I-*Bgl*II fragment (−47 to +51) to the *Xba*I-*Bgl*II cut plasmid using the following oligonucleotides 5′-CTAGACTGCCTACG-3′ and 5′-TAGGCAGT-3′, which restores the *Xba*I and *Hha*I sites. This plasmid without a CCAAT box was named D.R δCCAAT.

2.7 *Transient expression assays*

Human HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). The HeLa cells were transfected using DEAE dextran as the facilitator [24]. Briefly, two days prior to the transfection 2×10^6 cells were plated in 9-cm^2 dishes. The medium was removed, and DNA at 10 μg/ml was added in 2 ml of DMEM with 250 μg/ml DEAE dextran (Pharmacia, Piscataway, NJ) for 1 h at 37°C. This solution was removed, and the cells were osmotically shocked for 4 min at room temperature with 1 ml of 10% glycerol in DMEM. After removal of the glycerol solution and washing with 10 ml of DMEM, the cells were allowed to recover for 24 to 48 h in 5 ml of DMEM with 10% FBS. Then the cells were treated overnight with recombinant human α-interferon (Hoffmann LaRoche, Nutley, NJ) at 500 U/ml.

2.8 *Chloramphenicol acetyltransferase (CAT) assays*

CAT assays were carried out as described by Gorman et al. [25], using 100 μg protein of cell extracts and with 0.25 μCi of [^{14}C]chloramphenicol at 57 mCi/mmol (Amersham). % conversion was calculated as follows: (CPM of acetylated chloramphenicol/CPM of acetylated chloramphenicol + CPM of non-acetylated chloramphenicol) × 100.

3. RESULTS

3.1. *Structural characteristics of the 6-16 5′-flanking region*

A partial DNA sequence and important features of the upstream region are shown in fig.1. There is a

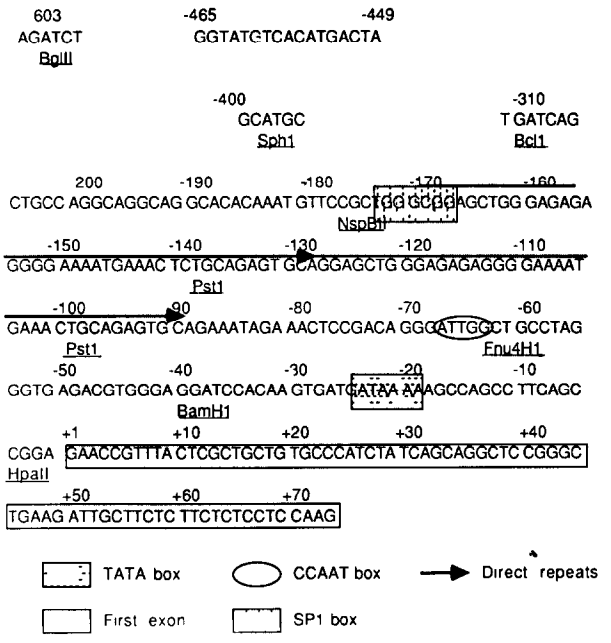

Fig.1 The 5′-flanking region of the 6-16 gene The main subregions of interest are indicated and are described in the text. Restriction sites used for subcloning are underlined

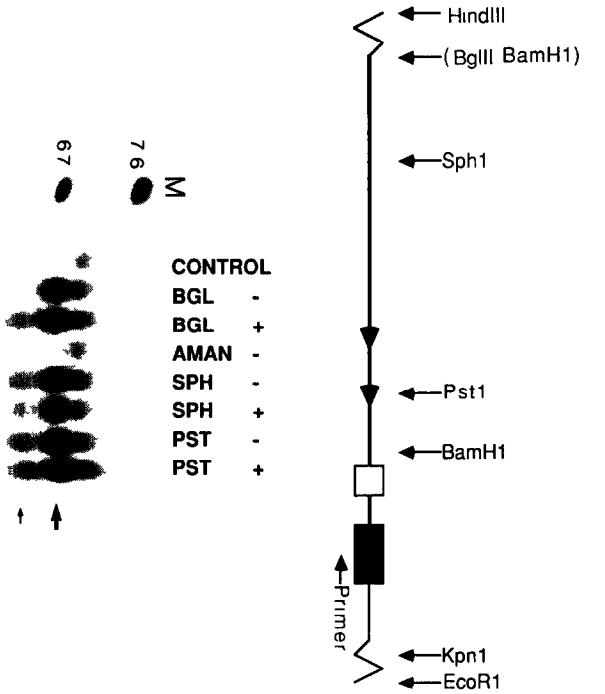

Fig 2 In vitro transcription of 6-16 and effects of 5′ deletions in the 5′-flanking region. The upper panel shows the 5′-flanking region of 6-16 schematically (not to scale). Horizontal arrows mark the position of the direct repeat units, the TATA box is shown in white and the black box is the first exon Wavy lines indicate the polylinker regions of pUC18 The 6-16 fragment used extends from the *Bgl*II site (−603) to the *Kpn*I site (+900) The 5′-end points of deletions generated by the use of *Bgl*II (−603), *Sph*I (−397) or *Pst*I (−96) are indicated The position of the 5′-labelled oligodeoxynucleotide primer used for reverse transcription is shown as an arrow The lower panel shows the labelled extended cDNAs, synthesized by reverse transcription from unlabelled in vitro transcripts Large and small arrows indicate major and minor products, respectively Control, a transcription reaction run without DNA; AMAN, a transcription reaction run with the *Bgl*II template in the presence of α-amanitin (0 5 μg/ml), (−), control extract; (+), extract from HeLa cells treated with 300 U/ml of α-interferon (Wellferon) for 4 h; markers, pBR322 digested with *Hpa*II

TATA box (ATAAAA), similar to the one upstream of the β-globin gene [26], located about 20 nucleotides upstream of the mRNA start site as mapped previously by primer extension [11]. The sequence −89 to −168 contains a tandem duplication in which the upstream 41 base pairs are repeated exactly, save for a deletion of two base pairs (compare position 99-100 with position 138-141). This region is clearly very important for regulation of transcription by interferon [1]. The tandem duplication is preceded by a potential site for Sp1 site (GC box) at position −167 to −172, but there is no indication that this site plays any role in the transcriptional regulation of 6-16 [1]. There is a CCAAT box at −63 to −67 in the non-coding (minus) strand, as are the CCAAT boxes of the Herpes simplex virus thymidine kinase gene [27] and the human HSP-70 gene [28]. Computer analysis of the 6-16 promoter region reveals several inverted repeats. One of the most stable of these ($\Delta G = -11$ kcal) can be represented as a structure in which the stem is formed by base pairing between nucleotides −146 to −134 and nucleotides −100 to −89, with most of the downstream (39 bp) unit of the tandem duplication in the loop formed by nucleotides −133 to −101.

3.2. *Transcription in vitro*

Soluble nuclear extracts from untreated or interferon-treated HeLa cells (freshly grown) were prepared as described by Dignam et al. [22]. A template that extends from 2.3 kb upstream (*Xho*I) to 0.9 kb downstream (*Kpn*I) of the transcription start site [11] was capable of directing correctly initiated transcription in vitro (data not shown). As shown in fig.2, transcription was initiated in vitro from a template which includes only 600 nucleotides of upstream sequence at the same position as for the endogenous gene in vivo. Deletion of the region between the *Bgl*II site at −603 and the *Pst*I site at −96 caused no significant change in the level or site of initiation of transcription in vitro, even though the tandem duplication was not present (fig.2). On the other hand, deletion to the *Bam*HI site at −39 abolished transcription completely (data not shown), showing that transcription is likely to be dependent on the CCAAT box at position −63 to −67. RNA polymerase II catalyzed the transcription in vitro since the reaction was inhibited by a low concentration of α-amanitin (fig.2).

We tested various templates for differential transcription in extracts of interferon-treated or untreated

cells and found that all of them were transcribed similarly in either type of extract (fig.2). The basal level of transcription was not decreased when the −449 to −465 region or the tandem duplication were deleted. Active transcription of the 6-16 gene in extracts of untreated cells contrasts strongly with the situation in vivo. We have tried in many experiments to detect differential regulation in vitro by using an approach similar to that of Wildeman et al. [29], varying the concentrations of Mg^{2+}, spermidine and DNA (50 to 500 ng). Still, no differential effect was seen (Chernajovsky, Yu., unpublished).

3.3. *Deletion of the CCAAT box abolishes basal and inducible transcription*

It was shown that the interaction in vitro of the direct repeat with nuclear proteins occurs also in extracts of untreated cells (figs4,6). Therefore we wanted to know if that interaction seen in vitro represents the binding of a transcription factor which could serve to maintain a basal level of transcription. Therefore we constructed an interferon-inducible promoter by cloning the direct repeat element of 6-16 into the reporter plasmid pBLCAT8+ (fig.3). The plasmid obtained named D.R.pBLCAT8+ was interferon activatable (table 1).

Table 1

Regulated expression by interferon requires the direct repeat and basal expression requires the CCAAT box

Plasmid	Treatment	% Conversion
pBLCAT8+	none	9 79
	αIFN	4 4
D R pBLCAT8+	none	5 6
	αIFN	53 7
D R.δCCAAT	none	0 3
	αIFN	0 3

HeLa cells were transfected with the indicated plasmids and after 48 h the cells were extracted for CAT assay. % conversion of the chloramphenicol was assessed as explained in section 2 αIFN, α-interferon

In order to test if the direct repeat element could serve as a basal transcription element, we deleted a region of the promoter of the HSV tk promoter containing the CCAAT box (from −104 to −47). Table 1 shows that this plasmid D.R.δCCAAT is devoid of transcriptional activity, both at basal level and after the cells are treated with α-interferon, showing that the CCAAT box is essential for the basal and the inducible-enhancer activity of the direct repeat and that the direct repeat cannot serve as a basal transcription element.

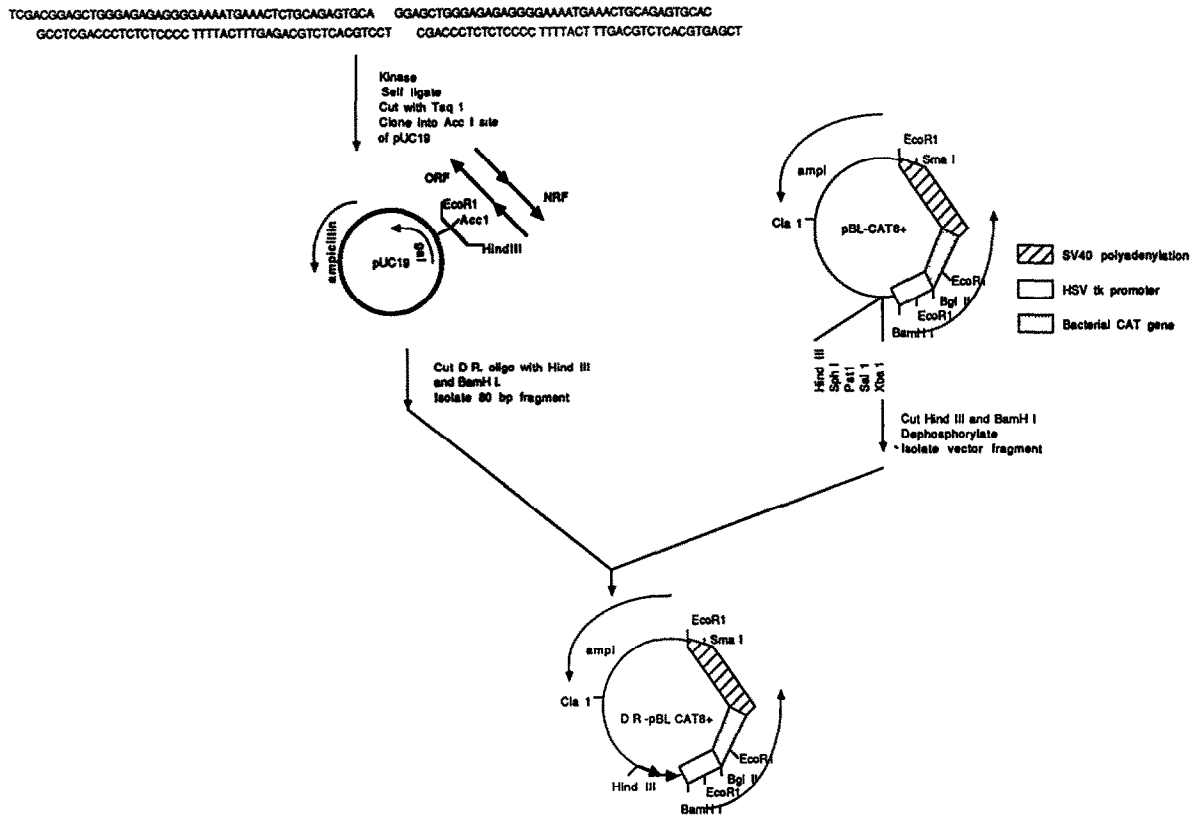

Fig.3. Construction of the interferon-inducible plasmid D R pBLCAT8+ The direct repeat of the 6-16 promoter was first cloned from two oligonucleotides into pUC19 and then the *Bam*HI-*Hin*dIII fragment from the plasmid containing the insert in an open reading frame with respect to β-galactosidase was subcloned into pBLCAT8+ (see section 2)

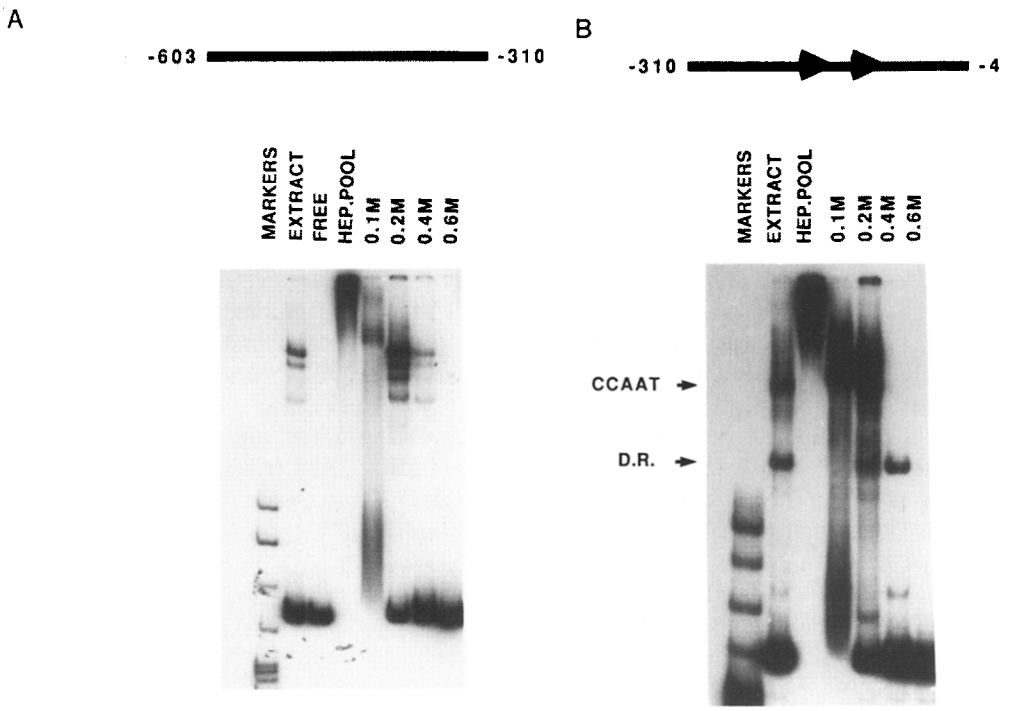

Fig.4. Detection and partial purification of constitutive nuclear factors that bind to the 6-16 promoter region. Transcriptionally active fractions from heparin-agarose columns (see the text) were chromatographed on DEAE-Sepharose columns. Increasing step concentrations of KCl were used for elution, as shown by the numbers above each track (0.2 M corresponds to 0.225 M). The bars at the top of each panel indicate DNA fragments corresponding to different 5′-flanking regions. (A) End-labelled DNA was incubated with 5 μl of each fraction. The protein concentrations were determined by the procedure of Bradford [42]; they were as follows: control: hep. pool, 2 mg/ml; 0.1 M, 0.2 mg/ml; 0.225 M, 1.2 mg/ml; 0.4 M, 0.5 mg/ml; 0.6 M, 0.1 mg/ml; extract, control total nuclear extract; hep. pool, pooled fraction from the heparin-agarose column; free, DNA fragment incubated with buffer alone. (B) As in panel A, but using a different DNA fragment as indicated. The hep. pool fractions had an excess of protein to competitor poly(dI.dC), which led to retention of the retarded complexes at the origin. The marker DNA is from pBR322, digested with *Hpa*II. The different complexes resulting from protein binding to the CCAAT box and to the direct repeat (D.R.) are indicated by arrows.

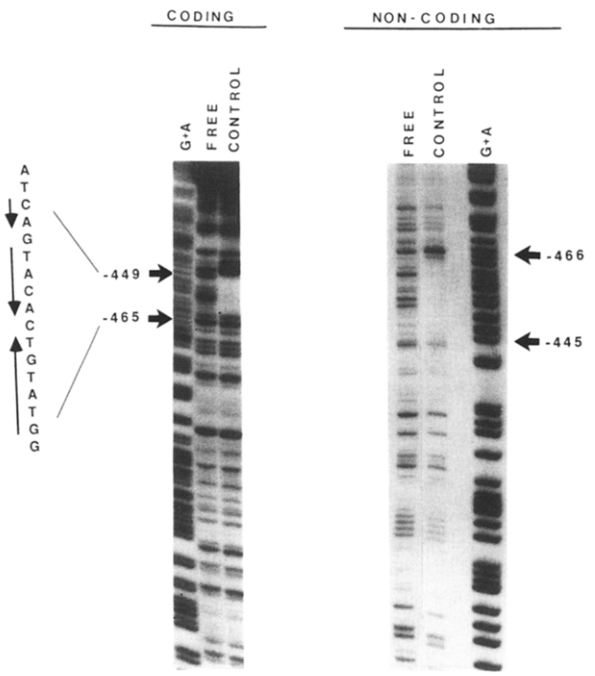

Fig.5. Protection from DNase digestion of a subregion of the −603 to −310 fragment. To obtain a labelled fragment corresponding to the coding strand, the restriction-cut *Hin*dIII site of the *ΔBgl*II clone was labelled at the 5′-end and the DNA was then cut with *Bcl*I (see section 2). The fragment corresponding to the non-coding strand was made in the same way, except that the end-labelled DNA was cut with *Hin*dIII. The tracks labelled G + A show the sequence ladders for the two fragments [43]. Complexes formed with the proteins eluted from DEAE-Sepharose in 0.225 M KCl, derived from extracts of untreated or interferon-treated cells, were digested with DNase I and then separated in a gel. They were analyzed in 6% acrylamide-urea sequencing gels. The boundaries of the protected regions are shown and the dyad symmetry of the protected site is indicated by arrows.

3.4. *Detection and partial purification of nuclear factors that bind to the 6-16 promoter region*

We searched for nuclear proteins that could bind to various regions of the gene using the band-shift method (gel retardation assay) of Garner and Revzin [30]. The *Bam*HI fragment (−39 to +437), which includes the TATA box, the first exon and part of the first intron, failed to form any retarded DNA-protein complex (not shown). Retarded complexes were detected using DNA

fragments corresponding to two different upstream regions of the 6-16 gene, one from *Bgl*II to *Bcl*I (−603 to −310) and a second from *Bcl*I to *Hpa*II (−309 to −5). We fractionated the HeLa nuclear extracts chromatographically (fig.4A), using the first two steps described by Dynan and Tjian [31] for purification of the transcription factors Sp1 and CTF [32]. Extracts were first loaded on a heparin-agarose column and the transcriptionally active fractions which were eluted in 0.3 and 0.4 M KCl were diluted and placed on a DEAE-Sepharose column at 0.1 M KCl. Proteins were eluted from the DEAE-Sepharose column by increasing salt concentration steps. Sp1 and CTF were eluted in 0.1 M KCl [32]. The nuclear protein(s) that bound to the −603 to −310 region co-eluted in 0.225 M KCl. They formed a complex pattern of retarded bands (fig.4A). The nuclear protein(s) that bound to the −309 to −5 region could be partially separated into two fractions (fig.4B). Protein(s) that formed the lower complex were eluted in 0.1 M and 0.225 M KCl, while protein(s) that formed the lower complex were eluted in 0.225 M and 0.4 M KCl. We also observed very similar complexes when extracts from untreated Daudi lymphoblastoid cells were used (not shown).

3.5. *Definition of the DNA sequences that interact with nuclear protein(s)*

The DNase-footprinting method of Galas and Schmitz [33] was used, with end-labelled DNA fragments and protein fractions from the DEAE-Sepharose column. After digestion with DNase, the DNA-protein complexes were separated by electrophoresis and extracted from the gel. Protected regions were detected by denaturing the DNA and analyzing it on sequencing gels. The nuclear protein(s) that produced the upper and most intense complex with the −603 to −310 fragment (fig.4A) protected a region from −449 to −465 which contains an almost perfect palindromic sequence (fig.5).

The upper complex, formed with the −309 to −5 fragment and nuclear protein(s) that were eluted in the 0.1 M and 0.225 M KCl fractions of the DEAE-Sepharose column, showed protection of the region of the CCAAT box (fig.6), suggesting that the nuclear protein(s) involved are similar to CTF, purified by Jones et al. [32]. The lower complex, formed with the

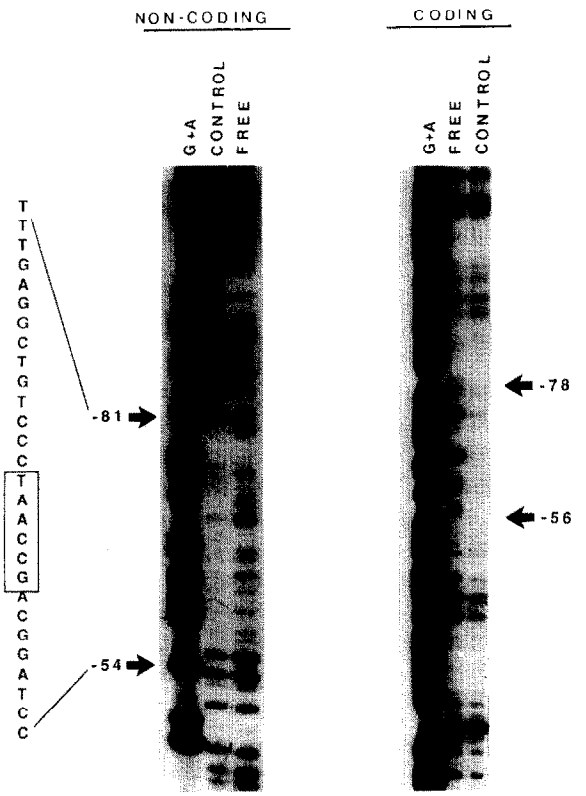

Fig.6. Protection from DNase digestion in the upper complex formed with the subregion of the −309 to −5 subfragment. To footprint this region, the *Asp*718 site of plasmid *ΔBgl*II-*Hpa*II (in the polylinker) was end-labelled with polynucleotide kinase (non-coding strand) or with reverse transcriptase (coding strand) and then cut with *Bcl*I. The upper complexes formed with this fragment and the 0.225 M KCl fractions from DEAE-Sephadex columns were isolated and analyzed as in fig.4. The tracks labelled G + A show the sequence ladders for each strand [43]. The boundaries of the protected regions and the corresponding DNA sequence are shown.

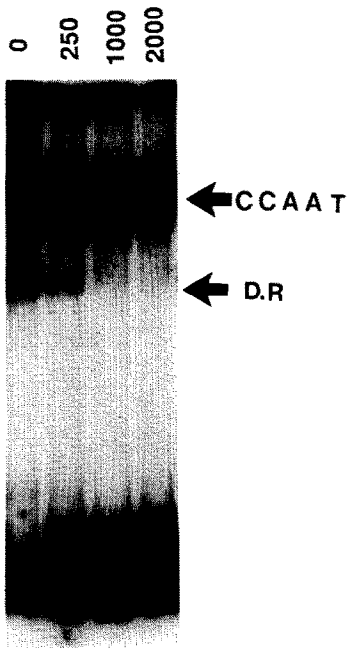

Fig.7. The lower protein-DNA complex in the −309 to −5 region is due to binding to the direct repeat region. Band shift assay using the −309 to −5 fragment (1 ng of labelled DNA) and nuclear extracts (1 μg of protein) of HeLa cells. Formation of the lower complex was competed for by increasing concentrations of a double-stranded oligodeoxynucleotide corresponding to the 41 nucleotides of the 5′-unit of the tandem duplication plus 4 nucleotides of adaptor sequence. The numbers at the top of each track indicate the nanograms of oligodeoxynucleotide used as competitor. Arrows show the complexes formed with the CCAAT and direct repeat (D.R.) elements. Similar results were obtained using a synthetic oligodeoxynucleotide corresponding to the downstream unit of the tandem duplication (data not shown).

same DNA fragment and protein(s) that were eluted in the 0.225 M and 0.4 M KCl fractions, involves binding to the direct repeat region of the promoter, as shown by competition with a synthetic oligodeoxynucleotide corresponding to the 41 nucleotide upstream copy of this repeat (fig.7). Attempts to footprint the lower complex with DNase were unsuccessful, even when the experiments were conducted under conditions optimal for footprinting the CCAAT box. The lower complex was reproducibly hypersensitive to DNase and disappeared after digestion, even when a large excess of protein was used. It seems most likely that the interaction between the direct repeat and the nuclear protein(s) that binds to it is relatively weak and that the caging effect of the acrylamide gel helped to stabilize the complex in the retardation assay. Indeed, in order to footprint the binding in this region, Dale et al. [34] had to use in situ orthophenantroline footprinting.

4. DISCUSSION

We have transcribed the interferon-inducible 6-16 gene in vitro and have found that a CCAAT box at position −63 to −67 is required for basal expression. In vivo transient transfection experiments with expression vectors have confirmed the essential role of the CCAAT box. Analyses by band shifting and DNase I footprinting showed that nuclear protein(s) with the chromatographic properties of CTF [32] bound to this region. The exact identity of this protein(s) is not known since it recently has been reported by Dorn et al. [35] and Santoro et al. [36] that the proteins that bind to CCAAT boxes seem to represent a family of transcription factors with different specificities. Nuclear proteins are also bound to the −448 to −465 region and to the tandem duplication at position −89 to −168. The latter sequences are clearly important in mediating the response of the 6-16 gene to interferon [1]. Pine and Darnell [37] have shown that the interferon-regulatory region of the ISG54 gene is DNase hypersensitive when the cells are not treated with interferon, suggesting that a nuclear factor, ISGF1, is bound to it. The interferon-regulatory element of ISG54 is identical to the interferon-regulatory element of 6-16 [1]. The constitutive complex of protein-DNA that is competed by the direct repeat oligonucleotide is most probably similar to the one formed by ISGF1. In our experience it has been impossible to get an interferon-modulated complex with the −310 to −5 promoter fragment in conditions in which such a complex could be seen with the direct repeat oligonucleotide [1] or with a cloned unit containing both units of the direct repeat (Chernajovsky, Yu., unpublished). This difference is probably due to the fact that the promoter fragment is too large or because the neighboring sequences of the direct repeat affect binding in vitro. Nevertheless, deletion of both the −449 to −465 and the −89 to −168 regions had no appreciable effect on the level of transcription in vitro or on the accuracy of initiation. Furthermore, transcription was as active with nuclear extracts from interferon-treated cells as with extracts from untreated cells, in striking contrast to the situation in vivo where the 6-16 gene is essentially silent in untreated cells. Similarly, when the early SV40 or the adeno major late promoter were transcribed in these extracts, the level of transcription was similar, showing that the transcriptional abilities of the extracts were equal (data not shown). There are many possibilities for why we did not observe correct regulation of transcription in vitro. For example, if negative regulation is important, there may not be an appreciable amount of free repressor protein in the nuclear extracts or the free DNA template may not be in an appropriate state to respond, compared with native chromatin. Alternatively, abnormal concentrations of transcription factors in the in vitro system may override control mechanisms. Probably the amount of the CCAAT binding protein is high enough to initiate transcription from the majority of the templates. Also, the interferon-treated extract used was from cells treated for 4 h with interferon, a time at which the peak of transcription is starting to decrease. Template usage is very poor in vitro; only about 1% of the DNA is transcribed, and the rest is trapped in non-functional protein-DNA complexes, perhaps competing with active complexes for transcription factors [31]. It is also possible that a labile post-translational modification of a regulatory protein, such as phosphorylation, may not be maintained in the nuclear extracts. It may be necessary to set up a reconstituted system in which the transcription factors can be added in varying amounts to distinguish among these and other possibilities.

The binding region at position −449 to −465 has dyad symmetry and is homologous to a region found in the interferon-regulated mouse gene 202 ([7], and see fig.6 in Porter et al. [1]). The protein-DNA interaction in this region is unlikely to play a major role in regulation of the 6-16 gene by interferon, since the region can be deleted without substantial effect [1]. Perhaps the 6-16 gene is regulated through the palindromic −449 tyo −465 region by stimuli other than interferon. Sequences with dyad symmetry are found in regulatory regions of many prokaryotic and eukaryotic promoters and probably indicate a binding site for oligomeric proteins [18,38,39]. The sequence TGACTA within this region is homologous to 5′-(C,G)TGACT(C,A)A-3′, a DNA element important for regulation by transcription factor AP1, and because the activity which binds to this element increases in cells treated with phorbol esters [40,41], protein phosphorylation may be important in regulation involving DNA elements such as these. We are investigating the function of the −449 to −465 region in vivo by linking it to a reporter gene.

Our results support the hypothesis that 6-16 is activated by a yet unknown mechanism that alters the affinity of ubiquitous proteins.

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