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An embryonic DNA-binding protein specific for a region of the human IFNβ_1 promoter

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ABSTRACT

Embryonal carcinoma (EC) cells are unable to make interferon in response to inducing agents. This block disappears after differentiation. We have found that nuclear extracts from undifferentiated P19 EC cells contain a DNA-binding activity which specifically recognizes a region within the human interferon-β_1 promoter. This activity is absent from differentiated cell types, both of EC and non-EC origin. The binding of the factor in undifferentiated EC cells leads to dramatic changes in the overall protein binding pattern of the interferon promoter as compared with differentiated cells, and may be responsible for repression of the endogenous interferon-β gene prior to differentiation.

INTRODUCTION

Embryonal carcinoma (EC) cells, the stem cells of the teratocarcinomas, have been used extensively as an in vitro system to study mammalian development and cell differentiation. The rationale for their use is the extensive similarity between these cells and those of the early embryo (1). The resemblence includes morphology, expression of a number of markers, and above all, pluripotentiality. EC cells will differentiate into a variety of tissue types both in culture and in tumours (1). Furthermore, some EC cell lines have been shown to be capable of contributing to all tissues in chimeric mice (1), thus demonstrating their functional totipotency.

A number of positive and negative markers for distinguishing EC cells from their differentiated derivatives have been described (1). These include a number of cDNA clones corresponding to mRNAs whose expression either increases or decreases during differentiation (2,3). To date little is known about the molecular mechanisms involved in the regulation of expression of these genes beyond identification of transcriptional and post-transcriptional components. The replication of a number of virus types is blocked or inefficient in EC cells (4,5,6). Although this appears to be a complex phenomenon, one component is certainly the decreased activity of the viral promoters in

the undifferentiated cells. In particular the enhancers of papovaviruses such as polyoma and SV40 and of some retroviruses seem to be inactive in EC cells. Evidence has been put forward for this being due to negative regulation in the undifferentiated cells and in some cases EC cell specific DNA-binding factors which recognize viral enhancer elements have been identified (7).

One of the negative markers for the undifferentiated state of EC cells is their inability to produce interferon (IFN) in response to exposure to viruses or double stranded RNA (8,9), a characteristic shared with early embryos (10). The IFNs are potent biological agents which exert pleiotropic effects on target cells or organisms (11). All of the IFN genes are strictly inducible with no expression being detected in untreated cells (11). Following exposure to inducers, transcriptional activation of the appropriate IFN gene or genes occurs. All differentiated cell types are competent for IFN induction. This also applies to both derivatives of EC cells and to differentiated cells of early embryos. Thus during cell differentiation a transition occurs from a state where IFN genes are incompetent to one where they can respond to induction (8,10). We have asked the question of whether or not differences could be found in the spectrum of DNA-binding proteins specific for the regulatory regions of an IFN gene when nuclear extracts from undifferentiated and differentiated cells were compared. We report that nuclear extracts from undifferentiated EC cells contain such a DNA-binding factor which is absent from differentiated cell types of both EC and non-EC origin. The binding of this factor to the HuIFNβ_1 promoter in EC cells leads to dramatic changes in the overall protein binding pattern of the region, as compared with differentiated cells, and may be responsible for repression of endogenous IFN expression prior to differentiation.

MATERIALS AND METHODS

Cells and plasmids

pIFNβ_1Hu was prepared by cloning a 1.8 kB EcoRI fragment with the coding and upstream sequences of HuIFNβ_1 into the polylinker of pGEM-1 (Promega Biotec, Madison, WI, USA). pHuIFNPr was prepared from this plasmid by subcloning the 282 bp EcoRI-HincII fragment containing the 5' upstream sequences of the gene into the polylinker of pGEM-1. Plasmids containing the DraI-AluI, AluI-AvaII, and DraI-AvaII fragments of this 5' region were a gift of John Hiscott.

Murine P19 EC cells were maintained as described previously (12) and differentiated with retinoic acid or DMSO according to the method of Rudnicki and McBurney (13).

Induction and titration of IFN production

Levels of IFN production were determined as described previously (14). For induction cells were exposed to Newcastle Disease Virus (NDV) (approximately 100 PFU/cell) for two hours. The virus-containing medium was removed

and fresh medium was added. The next day aliquots of this conditioned medium were removed, spun to remove cell debris, and added to cells in microtiter plates. The presence of HuIFN in the medium was detected by challenging the T98G cells with serial dilutions of Encephalomyocarditis (EMC) virus as described (15). MuIFN levels were determined using L929 cells challenged with vesicular stomatitis virus (VSV) (16).

Preparation of nuclear extracts

Nuclear extracts were prepared according to the method of Dignam et al. (17) from L cells, undifferentiated P19 cells, and P19 cells differentiated with DMSO. The extracts were aliquoted and stored in liquid nitrogen. Protein determinations were done using the Bio-Rad Protein assay kit (BioRad, Missaussauga, Ont., Canada).

Gel retardation assay

A fragment of the 5' non-coding region of the HuIFNβ_1 gene was end-labelled with ^{32}P-ATP (18) and mixed with 10 µg of nuclear extract in the presence of 250 ng HinfI digested pBR322, 6 µg poly (dI-dC)-poly (dI-dC), 1 mM DTT, 25 mM Hepes, pH 7.9, 40 mM NaCl, 1 mM EDTA, and 5% glycerol (for a total volume of 25 µl). pGEM-1 contains a HindIII site in the polylinker adjacent to the HincII site used for cloning, and in some cases this alternate site was used in preparation of the end-labelled fragments. For certain experiments synthetic oligonucleotides (prepared by G. Boileau, Université de Montréal) or subfragments of the promoter region were included to serve as specific competitor DNAs. The reaction mixture was incubated for 25 minutes at 25°C, following which 3 µl blue II sample buffer (18) was added and the samples were electrophoresed on 5% polyacrylamide TGE gels (50 mM Tris, pH 7.5, 380 mM glycine, 2 mM EDTA). After the samples had migrated sufficiently the gels were dried and autoradiographed.

DNase footprinting

Nuclear extracts were incubated with end-labelled fragments as for the gel retardation experiments. At the end of the 25 minute incubation period digestion with DNase I was performed according to the method of Jones et al. (19), using 50 ng DNase for samples without nuclear extract and 100 ng for samples with extract. The samples were extracted with phenol/chloroform, ethanol precipitated, and run on 5 or 8% sequencing gels. Lanes containing Maxam-Gilbert G+A reactions were included on each gel for calibration purposes but are not shown in the figures.

RESULTS

Expression of the human IFNβ_1 gene in P19 cells.

Since genomic clones of the murine IFNβ (MuIFNβ) gene were not available to us at the time we started this work, we first ascertained whether or not the heterologous HuIFNβ_1 gene was regulated appropriately in murine EC cells. A 1.8 kb EcoRI genomic fragment containing 282 bp of upstream and 713 bp of downstream coding sequence (20) was subcloned into pGEM-1, then co-transfected into P19 EC cells with the vector pSVtkneoβ, which encodes resistance to the drug G418. A pool of G418 resistant colonies (approximately 250) was obtained and the ability of these cells to produce human and murine IFN in response to NDV was tested prior to and following differentiation. The production of human IFNβ_1 (HuIFNβ_1) is distinguished from murine IFNs by testing the protective effect of the media from induced cells on human cell lines, since

Transfected	Differentiated	Induced	HuIFN titer	MuIFN titer
-	-	-	ND	<6 IU
-	-	+	ND	<6
-	+	-	ND	<6
-	+	+	ND	57600
+	-	-	<6 IU	<6 IU
+	-	+	<6	870
+	+	-	<6	<6
+	+	+	150	57600

Table 1. Titration of HuIFNβ_1 and MuIFN produced by transfected P19 cells. P19 EC cells were transfected with the 1.8 kB EcoRI fragment containing the sequence for HuIFN-β_1 (including 282 bp of 5' upstream sequence and the entire coding sequence). The spent culture media from the cells (either undifferentiated or differentiated with retinoic acid) were analysed for the presence of IFN before and after induction by NDV. The presence of HuIFN in the media was measured by challenging T98G cells with EMC virus while MuIFN levels were tested by challenging L929 cells with VSV. ND, not done.

IFNs cross-react poorly between species (11). As can be clearly seen from the results presented in Table 1, no HuIFN expression was found in the undifferentiated EC cell population either before or after induction. Following differentiation a significant titer of HuIFN was detected only in media from the virus-treated cells. Differentiated cultures derived from transfected or control non-transfected populations produced high levels of MuIFN when treated with virus. A relatively low, but significant, titer of MuIFN was observed in the virus-treated undifferentiated cultures of the transfected population, but never in non-transfected cells. Possible explanations of this will be discussed below. However, we conclude that the exogenous human gene is controlled by the regulatory factors responsible for repression of expression of the endogenous MuIFN gene in EC cells and that the transfected EcoRI fragment contains all the information necessary for this regulation.

Nuclear factors which bind to the HuIFNβ_1 promoter region

Figure 1 shows the restriction map and a schematic representation of the 5'-upstream region of the HuIFNβ_1 gene, with the location of the putative inducible enhancer indicated (21). This EcoRI-HincII fragment was subcloned into pGEM-1 (forming pHuIFNPr) in order to facilitate the preparation of end-labelled fragments which were used to analyse the interactions of nuclear factors with this part of the gene. In order to determine whether DNA-binding factors specific for the IFN gene could be detected in nuclear extracts we carried out gel retardation experiments with subfragments of the cloned 5'-upstream region. EcoRI-DraI, EcoRI-AvaII, AluI-HindIII and HaeIII-HindIII

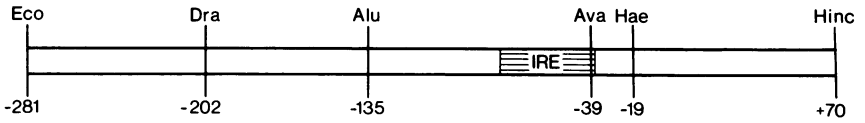

Figure 1. Schematic diagram of the 5' non-coding region of HuIFNβ$_1$. The numbering system of Zinn et al. (27) is used and indicates position relative to the mRNA cap site. IRE = Interferon Regulatory Element (22). Eco = EcoRI, Dra = DraI, Ava = AvaII, Alu = AluI, Hae = HaeIII, Hinc = HincII. The HincII site is immediately 5' to the ATG translation start site. The HindIII site in the pGEM-1 polylinker is 15 bp 3' of the HincII insertion site.

fragments were each tested for reactivity with nuclear extracts from L cells, which are highly competent for induction of their endogenous IFN genes (the HindIII site is only 15 nucleotides away from the HincII site in the pGEM-1 polylinker, thus digestion with either HindIII or HincII gives virtually identical restriction fragments). The EcoRI-AvaII and AluI-HindIII fragments both demonstrated a strong band of reduced mobility (Figure 2, lanes 4 and 7). These two fragments contain an overlapping sequence (corresponding to positions -135 to -39) which contains elements previously implicated in transcriptional control of the IFNβ gene (21,23). The shifted bands could be competed

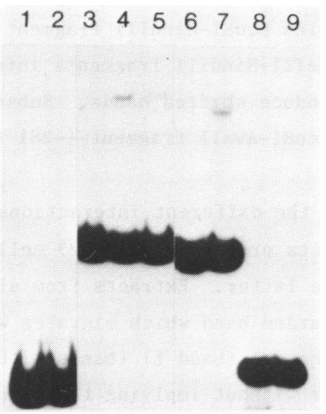

Figure 2. Nuclear factors interacting with subregions of the 5' untranslated region of HuIFNβ$_1$. The EcoRI-DraI (lanes 1,2), EcoRI-AvaII (lanes 3-5), AluI-HindIII (lanes 6,7) and HaeIII-HindIII (lanes 8,9) fragments were each end-labelled and tested for reactivity with nuclear extracts from L929 cells, as described in Materials and Methods. Lane 5 also contained unlabelled EcoRI-HindIII fragment (50 μg) added as a specific competitor. Bands were visualized by autoradiography after electrophoresis of the samples through a low ionic strength gel.

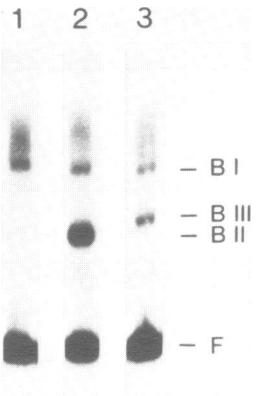

Figure 3. Nuclear factors interacting with the 5' non-coding region of HuIFNβ_1. Nuclear extracts (10 µg) prepared from L929 cells (lane 1), undifferentiated P19 cells (lane 2), or P19 cells differentiated with DMSO (lane 3) were incubated with the end-labelled EcoRI-AvaII fragment. B I, B II, and B III indicate retarded bands I, II, and II, respectively; F indicates the unbound fragment.

by the addition of unlabelled EcoRI-HindIII fragment (Figure 2, lane 5). Neither the EcoRI-DraI nor HaeIII-HindIII fragments interacted sufficiently with the nuclear extracts to produce shifted bands. Subsequent experiments were performed using only the EcoRI-AvaII fragment (-281 to -39 bp upstream of the mRNA cap site).

Figure 3 illustrates the different interactions between the EcoRI-AvaII fragment and nuclear extracts prepared from L929 cells, P19 cells, or differentiated derivatives of the latter. Extracts from all three cell types lead to the appearance of a retarded band which migrates with the same relative mobility near the top of the gel (band I) (bands with common mobility are assigned the same band number without implying that the same factors are bound to the probe in each case). In lane 2, where the probe was reacted with an extract from P19 cells, a strong band of faster mobility (band II) was also seen. Band II is not detected with extracts from the nuclei of L929 cells (lane 1), 3T3 fibroblasts (not shown) or differentiated P19 cells (lane 3). However, with nuclear extracts from the latter a different band of somewhat lower mobility (band III) is obtained. Band III is usually less intense than

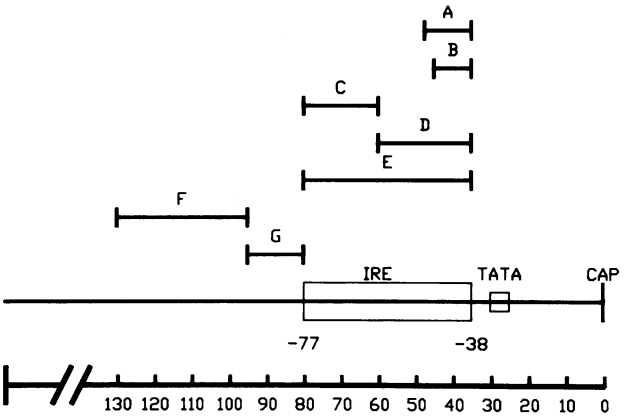

Figure 4. Relative positions of synthetic oligonucleotides used as competitors in gel retardation and DNase footprinting assays. The location of each of the oligonucleotides relative to the mRNA cap site is as follows: A (-47 to -35), B (-45 to -36), C (-79 to -64), D (-61 to -36), E (-79 to -36), F (-129 to -93) and G (-94 to -77). The locations of the cap site, TATA box, and IRE are also indicated.

band II, but this was found to be somewhat variable. The net result of this experiment is to show that a nuclear factor which specifically recognizes the HuIFNβ_1 promoter is present in EC cells (band II) and absent from fibroblasts and differentiated derivatives of EC cells. We have designated this factor as ECIF-1. Differentiated EC cells in turn contain a different DNA binding factor (band III).

A series of synthetic oligonucleotides derived from the HuIFNβ_1 upstream sequence were used as specific competitors in the gel retardation assay to further localize the interaction(s) of nuclear factors with the EcoRI-AvaII fragment. The relative positions of the oligonucleotides, which span the region from -129 to -38, are illustrated schematically in Figure 4. The shifted bands from all three extracts could be competed most efficiently with oligonucleotide F, corresponding to the sequence between -129 and -93. Results for extracts from undifferentiated and differentiated P19 cells are shown in Figure 5. In addition, the bands from extracts of undifferentiated P19 cells showed partial competition by oligonucleotide G while the bands from the differentiated P19 extracts were slightly inhibited by oligonucleotide E (-79 to -36), the region identified by Goodbourn *et al.* (21) as corresponding to the inducible enhancer of the HuIFNβ_1 gene (IRE).

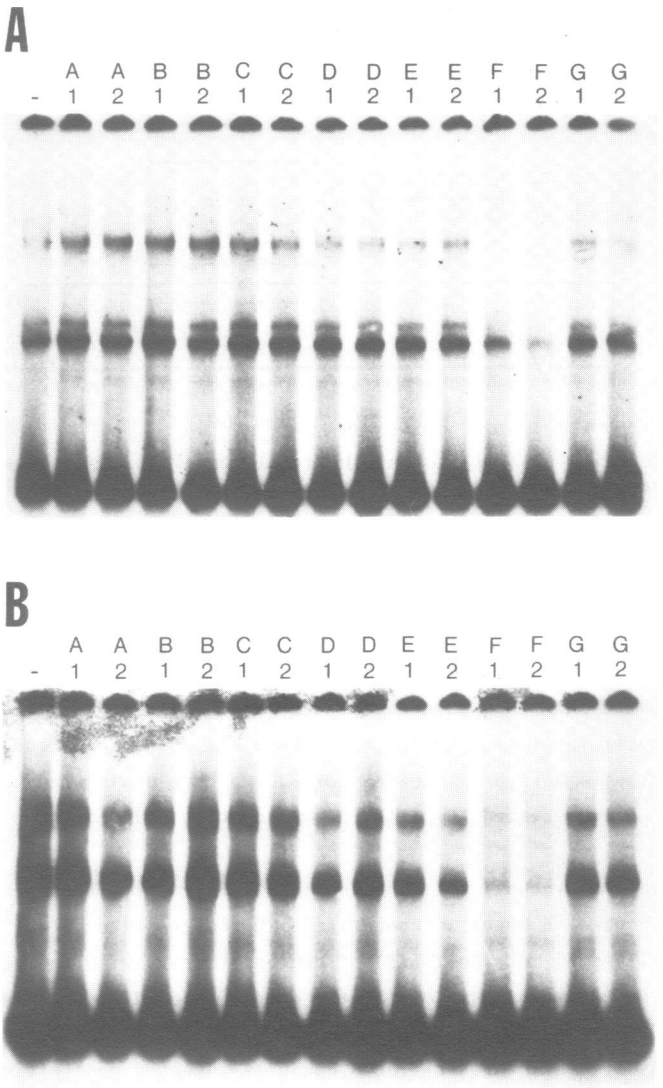

Figure 5. Gel retardation assay with P19 nuclear extracts showing competition by synthetic oligonucleotides. Nuclear extracts from undifferentiated (A) or DMSO-differentiated (B) P19 cells were incubated with the end-labelled EcoRI-AvaII fragment in the absence (-) or presence of oligonucleotides A through G as indicated above each lane. Either 100 or 200 ng of each competing oligonucleotide was added as specified by lanes marked 1 or 2 respectively.

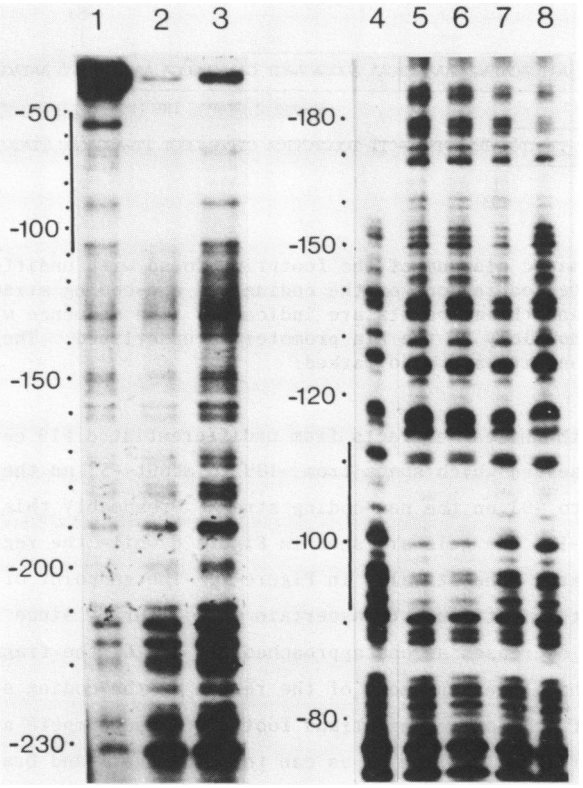

Figure 6. DNase footprinting assay with nuclear extracts from undifferentiated P19 cells. The EcoRI-AvaII fragment (labelled at either the 5' or 3' end) was incubated with nuclear extracts (20 µg) from undifferentiated P19 cells, subjected to DNase digestion, and electrophoresed on a sequencing gel. Lanes 1-3 show the DNase digestion pattern of the coding strand in the absence (lane 1) or presence of extract (lanes 2 and 3) and oligonucleotide F (lane 3). Lanes 4-8 show the noncoding strand digested with DNase in the absence (lane 4) or presence (lanes 5-8) of extract and fragments DraI-AluI, AluI-AvaII, and DraI-AvaII (lanes 6, 7, and 8 respectively). Numbers beside each gel mark the position relative to the mRNA cap site. Protected areas are indicated by a bar.

DNAse footprinting of the protein/DNA interactions

In order to better define the regions which bind factors from the three nuclear extracts under investigation, DNAse I footprinting experiments were performed. The EcoRI-AvaII fragment was labelled at either the 5' or 3' end to enable detection of interactions on the coding or non-coding strands re-

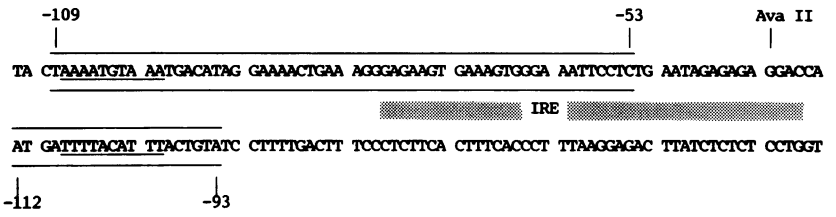

Figure 7. Schematic diagram of the footprint found with undifferentiated P19 cells. The protected regions on the coding and non-coding strands are enclosed by bars and the endpoints are indicated. The sequence within the footprint with homology to the Ela promoter is underlined. The IRE and the AvaII restriction site are also marked.

spectively. With nuclear extracts from undifferentiated P19 cells a protected region is observed which spans from -109 to about -53 on the coding strand and from -112 to -93 on the non-coding strand. Presumably this is due to binding of ECIF-1. The gels are seen in Figure 6 while the region of protection is illustrated schematically in Figure 7. The endpoint of the coding strand footprint is difficult to ascertain with accuracy since the resolution of the sequence decreases as one approaches the end of the fragment. Oligonucleotide F, which overlaps most of the region of the coding strand footprint and all of the non-coding strand footprint, can compete away the binding protein(s) (Figure 6, lane 3) as can the AluI-AvaII and DraI-AvaII fragments which also cover the region of the footprint (Figure 6, lanes 7 and 8). The DraI-AluI fragment, on the other hand, does not compete (Figure 6, lane 6).

In view of the footprints obtained with extracts from undifferentiated P19 cells two additional synthetic oligonucleotides were tested for their ability to compete the DNA-protein interactions. Oligonucleotide J corresponds to the region protected on the non-coding strand (i.e. -112 to -93) while oligonucleotide H corresponds to a shorter sequence (-108 to -99, underlined in Figure 7). Oligonucleotide J exhibited good competition of retarded bands in gel retardation experiments with nuclear extracts from both undifferentiated and differentiated P19 cells (Figure 8 and results not shown) while oligonucleotide H, shorter by only 10 base pairs (four from the 5' end and six from the 3' end), did not compete at all.

The footprints observed with nuclear extracts from differentiated cells (either L cells or DMSO-treated P19 cells) (Figure 9) are strikingly different from those observed with extracts from undifferentiated P19 cells. A large

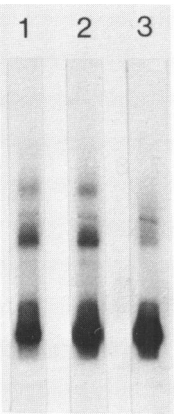

Figure 8. Gel retardation assay with nuclear extracts from undifferentiated P19. Nuclear extracts from undifferentiated P19 cells were incubated with the end-labelled EcoRI-AvaII fragment in the absence (lane 1) or presence of oligonucleotide H (lane 2) or J (lane 3). Oligonucleotide H comprises the sequence -108 to -99 while oligonucleotide J spans -112 to -93.

region of interaction on both the coding and non-coding strands was found with nuclear extracts from these cells, stretching from approximately -210 to -70 (as mentioned above the exact endpoints of the footprint cannot be accurately determined due to difficulty with resolution of the sequence at the ends of the fragment). The entire footprint can be competed by the DraI-AluI, AluI-AvaII, and DraI-AvaII fragments (Figure 9) as well as by oligonucleotide F (Figure 10). The most efficient competitors, DraI-AluI and oligonucleotide F, cover only a small portion of the protected sequence. This suggests that the cooperative binding of more than one protein is occurring in these differentiated cells.

DISCUSSION

The induction of the IFN genes has been shown by several groups to involve cis-acting sequence elements located within the first several hundred base pairs of the capping site and in all probability trans-acting proteins which interact with them. Both positive and negative regulation have been described, and an inducible enhancer has been located between -77 and -37 of the HuIFNβ_1 gene (21). This same group also used in vivo DNAse footprinting to show the existence of two regions within the HuIFNβ_1 gene promoter which bind protein (24).

In addition to the "on/off" regulation of induction the type I IFN genes are susceptible to other forms of regulation. These include genetic regulation of the levels of IFN transcription obtained following induction (25,26) and a developmental regulation which leads to undifferentiated cell types being incompetent for IFN induction (8,9). In the first part of this publica-

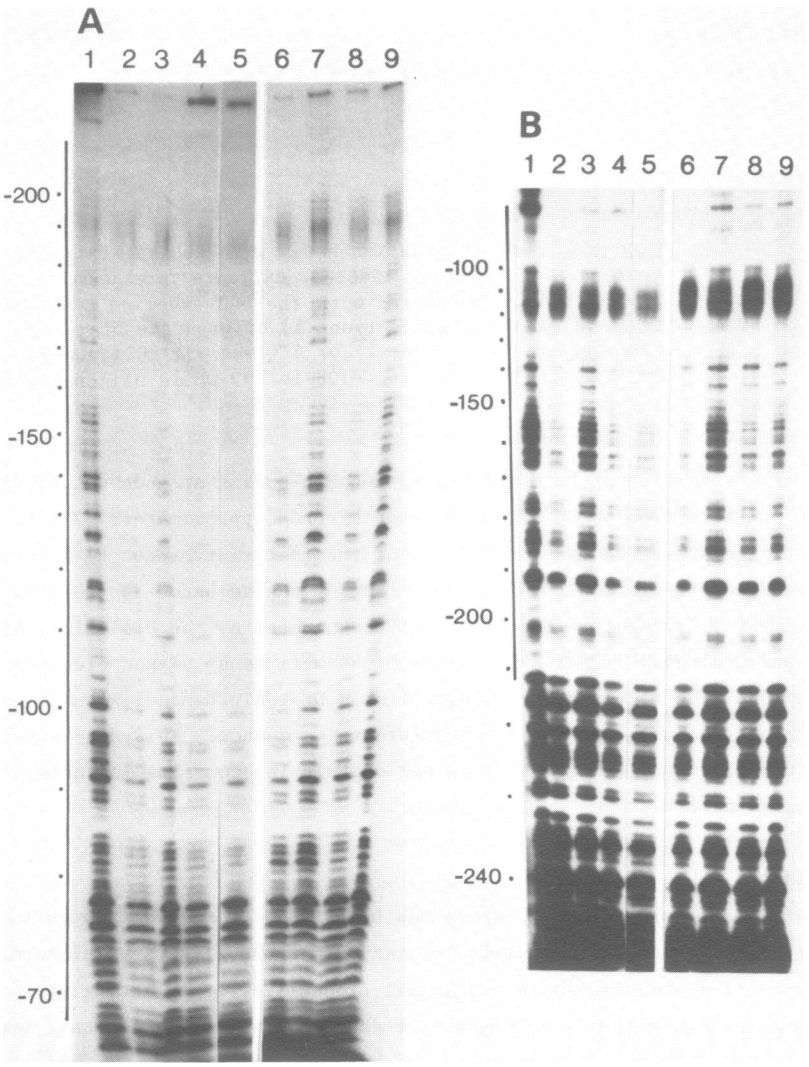

Figure 9. DNase footprinting assay with nuclear extracts from differentiated cells. The EcoRI-AvaII fragment (labelled at either the 5' or 3' end) was incubated with nuclear extracts from differentiated cells, subjected to DNase digestion, and electrophoresed on a sequencing gel. (A) Lanes 1-9 show the noncoding strand digested with DNase in the absence (lane 1) or presence of 20 µg of extract from L929 cells (lanes 2-5) or differentiated P19 cells (lanes 6-9). Competitions were performed using 400 ng of fragments DraI-AluI (lanes 3 and 7), AluI-AvaII (lanes 4 and 8), or DraI-AvaII (lanes 5 and 9). (B) Lanes 1-9 show the pattern of protection on the coding strand. Each lane contains the same extracts and competitors as in (A). Numbers beside each gel mark the position relative to the mRNA cap site. Protected areas are indicated by a bar.

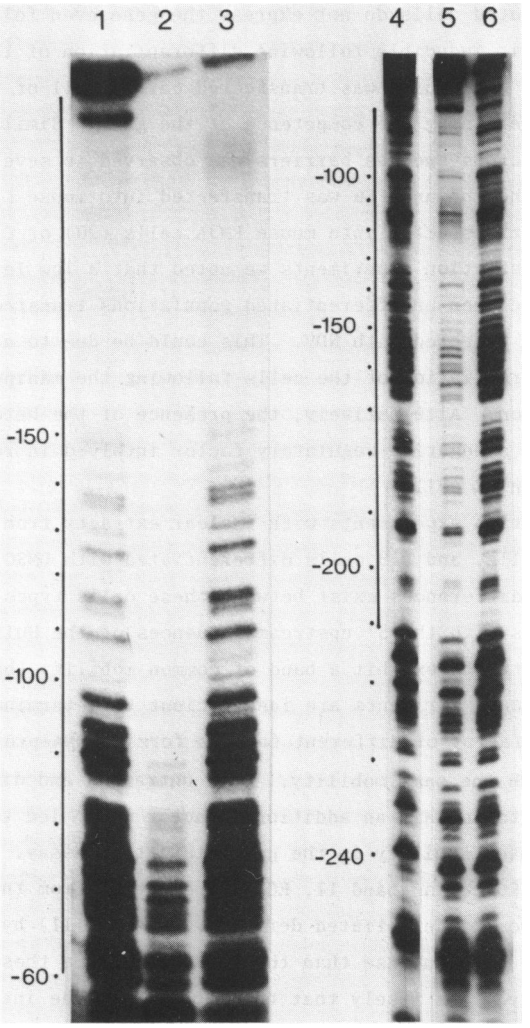

Figure 10. Inhibition of footprints by oligonucleotide F. The EcoRI-AvaII fragment was incubated with nuclear extracts from differentiated cells, subjected to DNase digestion, and electrophoresed on a sequencing gel. Lanes 1-3 show the DNase digestion pattern of the non-coding strand in the absence (lane 1) or presence of L929 nuclear extract (lanes 2 and 3) and oligonucleotide F (lane 3). Lanes 4-6 show the noncoding strand digested with DNase in the absence (lane 4) or presence (lanes 5-6) of nuclear extracts from differentiated P19 cells and oligonucleotide F (lane 6).

tion we have shown that the HuIFNβ_1 gene with 282 bp of upstream sequence, i.e. with all of the cis-acting elements implicated in the induction of the gene, shows the appropriate pattern of expression in murine EC cells (Table

I). Undifferentiated cells do not express the gene even following induction, whereas the gene is inducible following differentiation of the EC cells. Thus the region of the gene which was transfected carries all of the necessary information for determining the competence of the gene. Similar regulation of gene expression across species barriers was observed in several differentiated cell lines when human IFNα was transfected into mouse L cells (27) or when HuIFNβ_1 was transfected into mouse FM3A cells (20) or C127 cells (28).

In the transfection experiments we noted that a low level of endogenous MuIFN was detected when undifferentiated populations transfected with the HuIFNβ_1 gene were infected with NDV. This could be due to a low level of spontaneous differentiation of the cells following the manipulations involved in the transfection. Alternatively, the presence of the heterologous gene may have "titred out" a negative regulatory factor involved in repression of IFN gene expression in EC cells.

Gel retardation experiments with nuclear extracts from L cells, undifferentiated P19 cells, and P19 cells differentiated with DMSO, demonstrate that significant differences exist between these cells types with regard to their interactions with the 5' upstream sequences of the HuIFNβ_1 gene (Figure 3). All three extracts exhibit a band of common mobility (band I), although the gel retardation experiments are insufficient to determine whether this is in fact a common factor or different factors forming DNA-protein complexes which migrate with the same mobility. Both untreated and differentiated P19 cells were found to contain an additional factor which led to the appearance of a band of greater mobility in the gel retardation assay. However the complex obtained in EC cells (band II, ECIF-1) differed from that seen with nuclear extracts from differentiated derivatives (band III) by mobility, (and in some cases was more intense than the latter). Since these bands were of differing mobility it is likely that they are due to the interaction of different factors from the two extracts with the EcoRI-AvaII probe. The differences seen between L cells and DMSO-treated P19 cells in the gel retardation experiments may be partly due to the fact that they are different types of cells. The localization of regulatory sequences by other methods has been seen to depend, at least in part, on the type of cell under investigation (21,23,28,29).

Competition of gel retardation bands with synthetic oligonucleotides spanning the HuIFNβ_1 promoter region showed oligonucleotide F (corresponding to positions -129 to -93) to be the most efficient competitor (Figure 5). This region contains several interesting features: a region (-167 to -94)

shown by Zinn and Maniatis (24) to bind protein in vivo and a region which presents a high degree of sequence homology to part of the HuIFNα_1 (30) and IFNα_2 (21) promoters. This region also contains several of the hexamer repeats described by Fujita et al. (23) as important in obtaining full induction of the gene.

Further details of the DNA/protein interactions detected in the gel retardation experiments were obtained by DNAse footprinting. Extracts of undifferentiated P19 nuclei protected a relatively short region within the sequence of oligonucleotide F (Figure 6). The extent of the footprint is longer on the coding strand, reaching into the IRE, whereas it ends upstream of the IRE on the non-coding strand. In spite of the assymetric nature of the interaction, oligonucleotide F completely inhibits the interaction, although it contains no sequence from the extreme downstream part of the footprint. Furthermore, an oligonucleotide corresponding to the sequence which spans from -109 to -93, the part of the footprint which is protected on both strands, can alone compete efficiently for the interaction in both gel retardation and footprinting experiments. A shorter version of this sequence containing only the "box I" sequences (positions -108 to -99) is a much less efficient competitor: although it can compete in the footprint reaction (data not shown) it does not compete the gel retardation bands (Figure 8).

The DNAse footprinting pattern obtained with extracts from the nuclei of L cells or differentiated P19 cells is very different from that with the P19 extracts (Figure 9). The footprint covers a long region spanning from at least -210 to -70 and containing the region protected by extracts of undifferentiated cells. Some interruption of the footprint can be observed if the footprints are examined closely, indicating that multiple proteins may be involved. The patterns of the two types of differentiated cells are very similar. In both cases the whole footprint is competed out by oligonucleotide F (Figure 10), which represents only a small part of the total protected region. We interpret this to mean that cooperativity of binding exists, with occupancy of a binding site upstream of the IRE being obligatory for the binding of other factors. Other workers have provided evidence in related systems for functional cooperativity in this region. For example, Fujita et al. (23) have found that multiple copies of a hexamer repeat (homologous to the sequence AAATGT contained within this footprint) are necessary to confer inducibility on a reporter gene. Dinter and Hauser (31) found that duplication of the sequence from -90 to -51 resulted in increased IFNβ_1 expression after virus exposure. Kuhl et al. (30), using a similar repeated sequence found in the

IFNα1 promoter, found that multimers of the repeat are needed for inducibility and that monomers and dimers were insufficient.

The 5' end of the footprint (protected on both strands) also exhibits homology to a viral regulatory region: the sequence (GGAAGTGAAA) is partially homologous (5/10 bases the same) to the E1a enhancer core (32). Downstream, in the region protected only on the coding strand, is another sequence with even greater homology to the E1a enhancer core sequence (9/10 bases the same). The homology may be significant because it has also been shown that EC cells contain an activity which can replace the requirement for the adenovirus E1a product in replication of this virus (33,34). This activity, considered by some authors to be responsible for an enhancer-repressing activity (34), is lost upon differentiation.

It is tempting to speculate that ECIF-1 is a negative regulatory factor which binds to the IFN promoter in undifferentiated EC cells, preventing attachment of the alternate factors which bind here after differentiation. In the presence of ECIF-1 the gene is not capable of responding to induction. Several lines of evidence support this idea. The footprinting experiments show clearly that in undifferentiated cells only this site is occupied, as opposed to multiple sites in differentiated cells (a specific DNA-binding protein from undifferentiated EC cells has similarily been demonstrated in a retrovirus system (5)). Furthermore, we have found that cell fusions between EC cells and differentiated cells which normally are competent for IFN expression behave like the EC parent, and become inducible only if made to differentiate (unpublished results). It has also been shown that an enhancer-repressing (or E1a-like) activity exists in EC cells and that this activity disappears upon differentiation (33) supporting the involvement of a negative factor in the EC cell phenotype. One last curious point is that the sequence AAATGTAAA in the central region of the footprint is highly homologous to the sequence TAAATATAAAA, a functional part of a transcription silencer in yeast (35). In light of the increasing evidence for interchangeability of yeast transcription factors and those of higher eukaryotes (36,37), the similar sequences may indicate homology of function.

It is important to note that the results of gel retardation and footprinting experiments do not always coincide completely. The most notable example is seen where footprints using extracts from L cells or from differentiated P19 cells were almost identical, whereas in gel retardation experiments the latter extracts yield an additional band. It was also noted that certain oligonucleotides which did not compete in gel retardation did compete in the

footprint reaction. The relationship of the complexes observed in the gel retardation experiments to those giving rise to footprints is not entirely clear. Differences in gel retardation and footprint results are probably due to the different demands put on the stability of the complexes in the two techniques.

It is not possible at this time to deduce the relationship between the protein factors observed here and those detected by others or to determine the role of the varied protein binding regions observed in differentiated cells. Both positive and negative factors have been implicated in the induction of the HuIFNβ_1 gene in cells competent for IFN production (38,39,40). Our results suggest that when cells which are not competent for IFN production acquire the ability to be induced they undergo a concurrent alteration in the factors controlling the regulation of the IFN gene. At this point we do not wish to speculate on the possibility of changes in the pattern of factors following induction.

In conclusion we report here the existence of a DNA-binding factor in nuclear extracts of P19 EC cells which recognizes a specific sequence within the promoter region of the HuIFNβ_1 gene. This activity is absent in differentiated cells of both EC and non-EC origin. The factor, which we have called ECIF-1, shows some of the properties to be expected of a tissue specific repressor of transcription. Work is in progress to determine the regulatory nature of this protein and the cis-acting activity of its binding site.

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