Journal of Cellular Biochemistry 49:366–377 (1992)

Altered DNA/Protein Complexes Specific for the β-Interferon Regulatory Region Observed in Murine Embryonal Carcinoma F9 Cells

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Abstract Murine embryonal carcinoma (EC) F9 cells do not produce interferon (IFN) at the protein or RNA level in response to inducing agents, while retinoic acid differentiated F9 cells do produce IFN. A probe was constructed spanning positions −104 to −39 of the human β-IFN upstream regulatory region to examine this developmental control at the level of a transcriptional regulatory mechanism. Gel mobility shift analyses were used to examine this molecular mechanism to determine whether the differential expression of positive or negative trans-acting factors may act to control β-IFN expression in undifferentiated EC cells. These analyses showed that while nuclear extracts from poly-I,C induced L929 cells, in the IFN producing cell line, showed two shifted bands, nuclear extracts from both induced and uninduced F9 cells showed only one shifted band using the −104/−39 probe. While this single shifted band co-migrated with the faster migrating species of L929 cell extracts, competition analysis revealed differences between the two complexes. An oligonucleotide representing the positive regulatory domain PRDII competed efficiently for the probe when induced F9 cell extracts were examined, but failed to compete when induced L929 cell extracts were examined. In contrast, an oligonucleotide representing the positive regulatory domain PRDI competed very well when induced L929 cell extracts were examined but had only a minimal effect when induced F9 cell extracts were examined. These data suggest the involvement of developmentally regulated transcriptional factor(s) which have yet to be characterized.
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Key words: murine embryonal carcinoma, interferon, F9 cells

Embryonal carcinoma (EC) cells, which are the malignant, multipotential stem cells of a teratocarcinoma and show remarkable similarity to cells of the early embryo, have become a very useful model for studying the early stages of mammalian embryogenesis [Martin, 1980]. Changes in gene expression accompany the progression through the developmental process. While early-stage embryos are often inaccessible and large numbers of cells are unattainable, the EC cell system allows for the exploration of mechanisms that control gene expression during embryonic stem cell differentiation [Goodfellow, 1984]. Murine EC stem cell lines can be maintained as a stable population of undifferentiated cells which exhibit cellular and molecular properties characteristic of early embryos [Morello et al., 1982; Soltor and Knowles, 1978; Swartzendruber et al., 1977; Swartzendruber and Lehman, 1975]. In addition, some of these cell lines can be morphologically differentiated by in vitro treatment with retinoic acid [Hogan et al., 1981; Strickland and Mahdavi, 1978]. Accompanying the morphological changes are the associated changes in gene expression during the differentiation process [Soltor and Knowles, 1978; Kurkinen et al., 1983; Ozato et al., 1985; Rosenthal et al., 1984; Wang and Gudas, 1983]. Among the changes that occur are those involving expression of the interferon (IFN) system, which has been shown to be developmentally regulated during rodent embryogenesis [Barlow et al., 1984; Greene et al., 1984; Drasner et al., 1979; Gresser et al., 1984; Greene and Ts'o, 1986] as well as in several EC cell lines [Burke et al., 1978; Francis and Lehman, 1989]. In the early stages of development and in undifferentiated EC cells no response is made to numerous viral or synthetic IFN inducing

Received December 16, 1991; accepted March 13, 1992.

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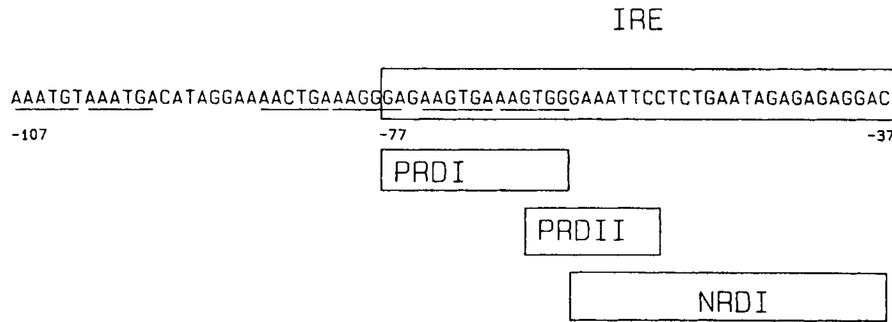

Fig. 1. Human β-IFN gene upstream regulatory region. Sequences from position −107 to −37 are shown with the interferon regulatory element (IRE) and its three regulatory domains in boxes. The hexamer sequence motifs, AAGTGA, are underlined.

agents. However, when the EC cells are triggered to differentiate over a period of days with retinoic acid treatment, the cells become susceptible to IFN induction which is detected at both the protein and RNA levels.

We and others [Francis and Lehman, 1989; Coveney et al., 1984; Haggarty et al., 1988; Harada et al., 1990] have suggested that the lack of β-IFN production in EC cells in response to an inducing agent may be controlled at a transcriptional level. To further investigate the developmentally regulated transcription of the IFN genes, it was necessary to examine events that control transcriptional activation of IFN in cells which respond to an inducing agent (viruses or polyinosinic-polycytidylic acid [poly-I,C]). Regulatory sequences and domains have been identified primarily in the human β-IFN gene by transfection analysis of numerous plasmid constructs in mouse fibroblasts [Ohno and Taniguchi, 1983; Zinn et al., 1983; Fujita et al., 1985; Goodbourn et al., 1985]. These combined studies of the Maniatis and Taniguchi laboratories defined a 40 bp inducible enhancer element, which was termed the interferon regulatory element (IRE) and encompasses a region from position −77 to −37 upstream of the transcriptional start site. Subsequently, the IRE was divided into three domains consisting of two positive regulatory domains, PRDI and PRDII, and one negative regulatory domain, NRDI [Goodbourn et al., 1986; Goodbourn and Maniatis, 1988]. The current model depicting the regulatory element and domains which control expression of the β-IFN gene is shown in Figure 1 [Fan and Maniatis, 1989].

Expression of the β-IFN gene is controlled by the binding of specific transcriptional modulators to *cis*-acting elements in response to the presence or absence of an IFN inducing agent in the extracellular environment. Several *trans*-acting factors interacting with the PRDI binding site have been identified [Zinn and Maniatis, 1986; Fujita et al., 1988; Keller and Maniatis, 1988; Miyamoto et al., 1988; Harada et al., 1989]. Interferon regulatory factor-1 and -2 (IRF-1 and IRF-2) have been isolated and identified as binding to the PRDI hexamer sequence motif, AAGTGA, which is also found four times in the 30 bp region immediately upstream of PRDI (Fig. 1). A number of factors have also been shown to bind to PRDII such as PRDII-BF [Keller and Maniatis, 1988; Fan and Maniatis, 1990] and NF-κB [Lenardo et al., 1988, 1989; Hiscott et al., 1989; Visvanathan and Goodbourn, 1989; Fujita et al., 1989]. Although several factors have been identified which bind to the β-IFN upstream regulatory region in IFN responsive cells, it is not known how any or all of these proteins function in cells that do not produce IFN such as undifferentiated EC cells.

In the present study, experiments were performed to examine protein interactions with the β-IFN upstream regulatory region which may be responsible for the developmental control of IFN expression at the level of transcription. Gel mobility shift and competition analyses examined β-IFN–specific DNA/protein interactions in the F9 EC cell system. Previously identified domains of the β-IFN regulatory region were used to assess β-IFN gene expression in the developmental model as compared to mouse L929 cells in response to the nonviral inducing agent polyinosinic-polycytidylic acid (poly-I,C). The results observed in these experiments suggest that a binding event critical for transcriptional activation of the β-IFN gene is absent in the undifferentiated F9 cell.

METHODS

Cell Culture

L929 (a murine fibroblast cell line), F9.22 (F9; an embryonal carcinoma cell line), and PYS-2 (parietal yolk sac endoderm cell line) were maintained by culturing the cells in Eagle's modified minimal essential media (MEM) containing twice the concentration of amino acids and vitamins and supplemented with 5% fetal bovine serum (FBS; M.A. Bioproducts), glutamine (2 mM), and antibiotics (100 units/ml penicillin and 80 μg/ml streptomycin). With normal tissue culture maintenance, consisting of subculturing every 3–4 days, F9 cells remained in the undifferentiated state. Differentiation into parietal endoderm was triggered by culturing the F9 cells in the presence of 0.5 μM all trans retinoic acid (RA; Sigma Chemical Co.). The differentiation process was monitored morphologically and by immunocytochemistry analysis as previously described [M.K. Francis, Ph.D. thesis, Albany Medical College, 1990].

Interferon Induction and Antiviral Determination

Subconfluent monolayers of the cells were treated with poly-I,C (50 μg/ml; Pharmacia) + DEAE-dextran (200–250 μg/ml, Pharmacia) in serum-free media for 2 h. Serum-free medium was used for mock inductions. Following a 2 h incubation with the inducing agents, the cell monolayers were washed with PBS (phosphate-buffered saline) and replated with MEM + 2% FBS. The cells and supernatants were examined at various times post-induction for the production of IFN. The supernatants were assayed for antiviral activity as described by Forti et al. [1986] and Francis [M.K. Francis, Ph.D. thesis, Albany Medical College, 1990].

Preparation of Nuclear Extracts

Each induced extract (+) was made from 3–12 × 10^7 cells induced with poly-I,C as described above. Uninduced extracts (−) were prepared from a corresponding amount of mock-induced cells. At 6 h post-induction, supernatants were collected and frozen at −20°C for analysis of antiviral activity and the cells were harvested by scraping in cold PBS. The cells were washed twice in PBS, counted, and stored as cell pellets at −20°C. Prior to the extraction, the cell pellets were quickly thawed at 37°C and placed on ice until further processing. All buffers used for the preparation of nuclear extracts were supplemented with 0.5 mM PMSF and 0.5 mM DTT. Nuclei were isolated as described by Higashi [1985]. Crude nuclear extracts were made as described by Dignam et al. [1983] with the exception that Buffer C contained 0.4 M NaCl. The extracts were dialyzed (5 h, 4°C) in Buffer D [Dignam et al., 1983] and small aliquots (30–50 μl) of the cleared dialysate were stored at −70°C. The protein concentration of the extracts was determined by the Bio-Rad Protein Microassay as per the manufacturer's description.

Plasmids

Following a *Hinc*II/*Eco*RI digestion of pBVIFΔ(−104) [Zinn et al., 1983; received from T. Maniatis], a 176 bp fragment from position −104 to +72 of the human β-IFN gene was isolated and subcloned into the multiple cloning site of pBS+ (Stratagene) to create pBS.−104HβIFN. An *Ava*II/*Eco*RI digest of this plasmid and radioactive labeling of the ends by a fill-in reaction using Klenow [Ausubel et al., 1989] resulted in the isolation of a 65 bp fragment (−104 to −39) which was used as the probe in the gel mobility shift assays. This 65 bp fragment and a 222 bp fragment of pBS DNA from this same plasmid were used as unlabeled competitors in these analyses. A *Bam*HI/*Bg*lII restriction digest of plasmids 11-6-2 and 2-7-1 (provided by T. Mamatis) resulted in fragments which were also used as unlabeled competitors. These fragments represented the 40 bp IRE from position −77 to −37 of the human β-IFN gene and a 24 bp quadruple repeat of the AAGTGA sequence motif of PRDI, respectively.

Synthetic Oligonucleotides

Oligonucleotides containing sequence motifs of the regulatory domains PRDI(PI), PRDII(PII), and NRDI(NI) and a non-specific sequence (JD5) were synthesized. The complementary strands were annealed at 85°C for 10 min and then cooled slowly to room temperature. The concentration of the annealed oligonucleotides was determined by comparison with known concentrations of molecular size markers after electrophoresis and ethidium bromide staining of a non-denaturing 10% polyacrylamide gel. The sequences of the fragments are as follows: PI 5′AAGTGAAAGTGAAAGTGAAAGTGA3′; PII, 5′GTGGGAAATTCC3′; NI, 5′CTCTGAATAGAGAG3′; and JD5, 5′GTCTTTCAGTCATATGG3′.

Gel Mobility Shift Analysis

Crude nuclear extracts were examined for the presence of DNA binding proteins which would interact specifically with an identified portion of the upstream regulatory region of the human β-IFN gene. The following binding mixture (15 μl) was incubated for 15 min at 30°C: 6–8 μg extract in 5 μl Buffer D, 4 μl supplemented Buffer D (0.985 ml Buffer D, 7.5 μl 0.5 M EDTA, pH 8.0, 3.8 μl 0.5 M $MgCl_2$, 3.8 μl 1 M DTT), poly-dI:dC (polydeoxyinosinic-polydeoxycytidylic acid (Sigma), 0.05–1 μg dH_2O), and 1 μl^{32}P-labeled −104 probe (0.4–1 ng/μl). Prior to addition of the probe, the other components were subjected to a 5 min incubation at room temperature. After adding 3 μl of Type III Sample Buffer [Maniatis et al., 1982] the samples were electrophoresed through a 4% non-denaturing polyacrylamide gel in the high ionic strength Tris-glycine-EDTA buffer as described by Ausubel et al. [1989].

Cold competition reactions were performed with unlabeled DNA fragments as an additional component of the binding reaction. The competitors were either annealed complementary synthetic oligonucleotides or plasmid fragments isolated after restriction enzyme digestion and separation on an 8% PAGE. The fragments were eluted from the gel in 0.5 M NH_4OAc, 1 mM EDTA according to Maniatis et al. [1982]. Concentrations of the unlabeled fragments were determined based on a known concentration of molecular size markers.

RESULTS

Since IFN production has been shown to be developmentally regulated, the murine EC cell line, F9, was used as a model to assess the control mechanism at the level of transcriptional activation of the β-IFN gene in response to the synthetic inducing agent, poly-I,C. Due to a strong homology (90–95%) between the mouse and human β-IFN regulatory region [Dirks et al., 1989] and the availability of plasmids containing various portions of the human upstream regulatory region, the human gene constructs were used to analyze nuclear extracts from induced or uninduced mouse L929 cells or F9 embryonal carcinoma cells for specific protein interactions with the β-IFN upstream regulatory region. Additionally, since variability defining the 5′ end of the regulatory region was observed with respect to the cell system analyzed [Zinn et al., 1983; Fujita et al., 1985; Goodbourn et al., 1986; Fan and Maniatis, 1989], a region larger than the 40 bp interferon regulatory element (IRE) was used to examine specific DNA/protein interactions involved in the developmental control of the transcriptional activation of the β-IFN gene.

The plasmid pBS.−104HβIFN, containing a portion of the human β-IFN gene from positions −104 to +72, was used to isolate a 65 bp fragment of the upstream regulatory region from positions −104 to −39 (−104 fragment). In addition to the laboratory constructed −104 fragment, other plasmid DNA fragments which represent the entire 40 bp IRE or a portion of it (4xHex) were used to address binding specificity to the larger fragment. The 4xHex is a 24 bp fragment isolated from plasmid 2-7-1 (provided by T. Maniatis) consisting of a quadruple repeat of the PRDI sequence motif, AAGTGA. Synthetic oligodeoxynucleotides (oligos) were also designed to represent the various domains of the regulatory region. The PI oligo, like the 4xHex fragment, is a four time repeat of the AAGTGA sequence motif. The PII oligo represents the NF-κB binding site in PRDII and the NI oligo is a centrally located sequence in the negative regulatory domain, NRDI. Finally, the JD5 oligo was obtained from J. Driscoll and was used as a non-specific DNA competitor. Each was annealed to an appropriate complementary oligo and used to determine which portion(s) of the β-IFN regulatory region is important for the binding of trans-acting factors in the murine EC cell system as compared to the inducible L929 cell system. Additionally, the EC cell culture model allowed for the consideration of the regulatory role of specific DNA binding proteins during the developmental process.

Analysis of L929 Cell Nuclear Extracts

Initially, the −104 fragment was used in gel mobility shift assays to examine extracts from uninduced and poly-I,C induced L929 cells for DNA binding proteins specific for this fragment. This was to establish a DNA binding standard with an IFN-inducible cell line. To determine the optimal quantity of carrier DNA, 6–8 μg of protein from nuclear extracts and 0.4 ng of end-labeled −104 fragment were mixed in a binding reaction with various concentrations of poly-dI:dC (carrier DNA; 50–1,000 ng; data not shown). Following electrophoresis and autoradiography, an appropriate amount of poly-dI:dC

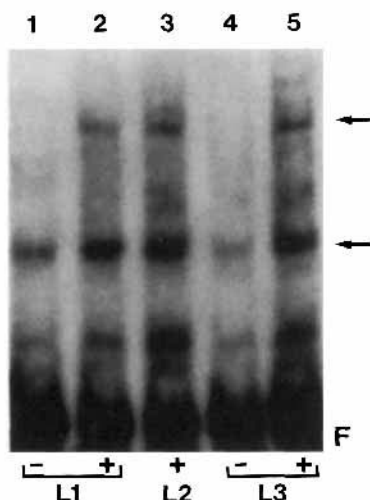

Fig. 2. Comparison of uninduced and induced L929 cell nuclear extracts by gel mobility shift analysis. Three L929 cell nuclear extracts (L1, L2, and L3, 6 μg each) from uninduced (−; **lanes 1, 4**) or induced (+; **lanes 2, 3, 5**) cells were assayed for binding to the −104. An amount of poly-dI:dC (1 μg) was used as carrier DNA in each reaction. Samples were electrophoresed through a Tris-glycine 4% polyacrylamide gel. F is free probe and shifted bands are indicated by arrows.

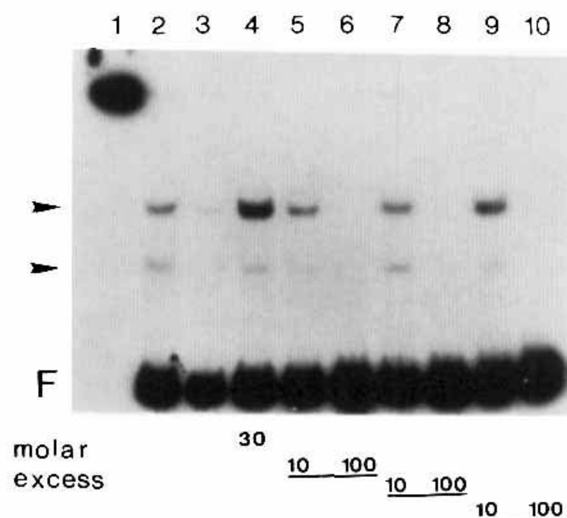

Fig. 3. Competition binding analysis of induced L929 cell extracts. Nuclear extracts from induced L929 cells (8 μg; Ll: **lanes 1, 2, 4–10;** L2: **lane 3**) and 0.4 ng of the −104 probe, 1 μg poly-dI:dC (lanes 2, 4–10; 0.5 μg in lane 3) were incubated with the indicated molar excess of competitor DNA fragments 222 (lane 4), −104 (lanes 5, 6), IRE (lanes 7, 8), and 4xHex (lanes 9, 10). Shifted bands are indicated by arrows and F is free probe.

was determined as the amount sufficient to prevent non-specific proteins from binding to the −104 probe, yet it did not inhibit the binding of proteins which recognize a specific sequence within the probe DNA. All extracts prepared throughout the course of this investigation were titrated with poly-dI:dC for this purpose. Three separate extractions (L1, L2, and L3) from mock-induced (−) and poly-I,C induced (+) L929 cells were examined by gel mobility shift analysis using the −104 probe (Fig. 2). While the assay revealed a single band of altered mobility with mock-induced extracts (Fig. 2, lanes 1, 4), two shifted bands were consistently detected (indicated by arrows) with extracts from poly-I,C induced L929 cells (Fig. 2, lanes 2, 3, 5). Upon longer exposure, no additional bands were observed with the mock-induced extracts (data not shown). Coincidentally, the faster of the two complexes from induced cell extracts migrated to the same position as the single species detected in extracts from mock-induced L929 cells (compare lanes 1 and 4 with lanes 2, 3, and 5 in Fig. 2). This may be indicative of identical proteins within the DNA/protein complex formed by the different extracts. This interpretation, suggests that a single steady state complex forms with the −104 fragment, while an additional, more slowly moving complex forms with cell extracts from poly-I,C induced cells. However, it is unclear if the two bands observed with induced L929 cell extracts represent different molecular weight proteins or multimers of one or more proteins binding to the probe to create the different mobilities.

To assess the binding specificity of the DNA/protein interactions observed with poly-I,C induced L929 cells, DNA competition assays utilizing molar excess amounts of unlabeled competitor DNA fragments were performed and the results are presented (Fig. 3). The competitor DNAs consisted of a non-specific DNA (222; lane 4), the entire 65 bp probe sequence (−104; lanes 5, 6), or portions of the probe representing the IRE (lanes 7, 8) and the PRDI sequence motif (4xHex; lanes 9, 10). Lanes 1 and 2 represent the normal binding reaction without and with, respectively, poly-dI:dC. The results show that unlabeled −104, IRE, and 4xHex fragments competed efficiently for the probe binding in both shifted bands. In fact, a tenfold molar excess of the 4xHex (lane 9) nearly abolished the lower band, while a hundredfold molar excess also competed for the upper band. The specificity of the reaction is shown in lane 4, where a thirtyfold molar excess of the 222 bp fragment did not compete for binding of the probe at either of the shifted bands. Since this fragment is 3.5 times larger than the probe, the thirtyfold

molar excess of fragment number corresponded to a hundredfold molar excess of nucleotide number, making the lack of competition more striking. These results suggested that the hexamer sequence, AAGTGA, may be responsible for both shifted bands with the −104 probe. Since the unlabeled 4xHex competed for both bands in a dose-dependent manner, our results suggest both high and low affinity interactions with this sequence. This experiment has been repeated with another L929 cell extract using ten-, twenty-, and fiftyfold molar excess of unlabeled competitors (−104 and IRE) which competed successfully for the two binding events visualized in the absence of competitor DNA.

Analysis of F9 Cell Nuclear Extracts

Since previous experiments by this laboratory [Francis and Lehman, 1989] had shown no detectable antiviral activity nor β-IFN RNA in F9 embryonal carcinoma cells in response to an IFN inducing agent, poly-I,C, the upstream regulatory region known to be involved in the transcriptional control of β-IFN was examined in the context of a developmental model. Nuclear extracts from poly-I,C induced and uninduced F9 EC cells, which produced no detectable antiviral activity, were compared with extracts from L929 cells (L1) which produced a detectable IFN titer of 48 U/ml (units per ml). Figure 4 is a gel mobility shift analysis of nuclear extracts from uninduced (lanes 3–6) or induced F9 cells (lanes 7–10) with the end-labeled −104 probe and varied amounts of carrier DNA (poly-dI:dC). Induced L929 nuclear extracts (L1) without and with carrier DNA (lanes 1 and 2, respectively) showed the two primary shifted bands from nuclear extracts of IFN-producing cells in response to poly-I,C. Although two bands were observed in both induced and uninduced F9 reactions without carrier DNA (lanes 3, 7), the upper band rapidly disappeared in the presence of carrier DNA (lanes 4–6, 8–10). These results suggested that only the lower band was due to a specific protein interaction with the −104 probe, which was in contrast to the induced L929 cell extracts which exhibited two shifted bands. The mobility shift analysis was repeated several times with this extract and a second induced F9 extract, confirming the appearance of a single band of altered mobility. This band migrated to the same position as the lower band that appeared in the analysis of the L929 cell extracts. While these results suggest that the DNA/protein complex observed in the F9 extracts may be the same as the faster migrating species in induced L929 cell extracts, it is possible that different proteins may have the same effect on altering the mobility of the labeled fragment. Additionally, since only a single complex was detected regardless of whether the F9 cells were induced, the results suggest that the lack of a particular DNA/protein complex may explain the transcriptional inactivity of the β-IFN gene in induced F9 cells.

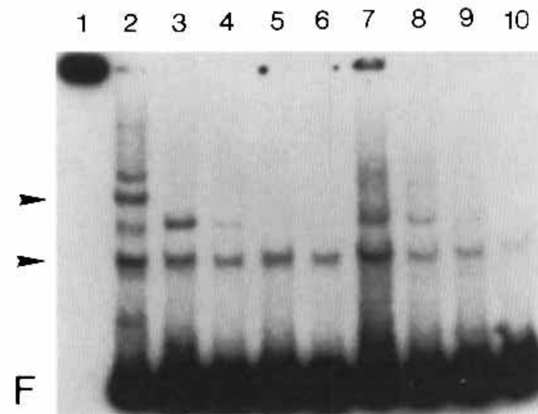

Fig. 4. Gel mobility shift analysis of F9 extracts titrated with poly-dI:dC. Nuclear extracts from induced or uninduced F9 cells were examined for binding to the −104 probe as described for Fig. 3. Uninduced (**lanes 3–6**) or induced (**lanes 7–10**) F9 extracts (8 μg) were incubated in the absence (lanes 3, 7) or presence of 50 ng (lanes 4, 8), 200 ng (lanes 5, 9), or 500 ng (lanes 6, 10) of poly-dI:dC L929 cell extracts (Ll; 8 μg) were incubated in the absence or presence of 1 μg poly-dI:dC (**lanes 1, 2**). Shifted bands are indicated by arrows and F is free probe.

To further assess the specificity of the DNA/protein interaction observed with induced F9 cell extracts in the gel mobility shift analyses, competition binding experiments were performed. In addition to the larger −104 and IRE plasmid fragments, annealed complementary oligonucleotides representing smaller portions of the regulatory region and a non-specific 17-mer were used in molar excess amounts as unlabeled competitors in the binding reactions (Fig. 5). Included in the competition binding analyses are examples of a specific, PI, (Fig. 5A) and a non-specific, JD5, (Fig. 5B) competitor. While the non-specific oligomer showed no competition at 500-fold molar excess (Fig. 5B), the PI oligomer representing the PRD1 sequence *motif* competed modestly at 200- and 500-fold molar excess. In addition, the PII oligomer representing the NF-κB binding site of PRDII competed very well even at fiftyfold molar excess (data not

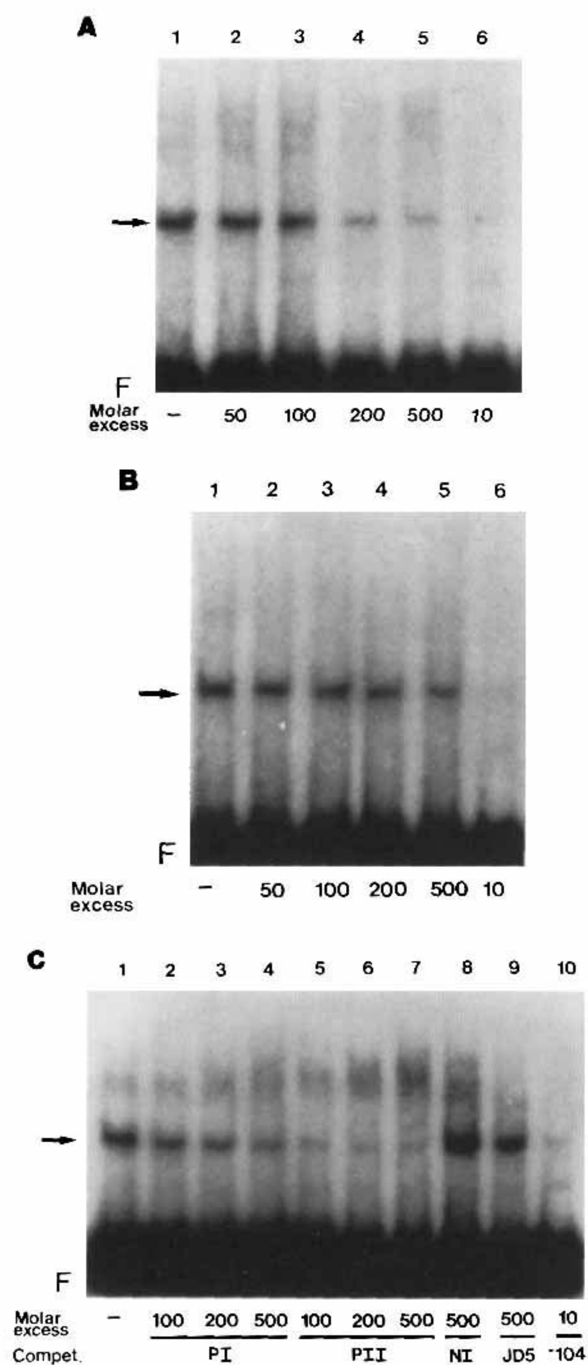

Fig. 5. Competition binding analysis of nuclear extracts from induced F9 cells. Induced F9 extracts (6 μg) were incubated in the absence (**lane 1**) or presence of the indicated molar excesses of the competitor oligonucleotide and 1 ng of the −104 probe. PI (**A**) or JD5 (**B**) are shown as examples of specific (PI) and non-specific (JD5) competitors. A tenfold molar excess of unlabeled −104 fragment is included (lane 6) for both A and B. Selected molar excesses (**C**) of PI (**lanes 2–4**), PII (**lanes 5–7**), NI (**lane 8**), JD5 (**lane 9**), and unlabeled −104 fragment (**lane 10**) were compared. The shifted bands are indicated by arrows and F is free probe.

shown). In addition to the titration of each competitor, tenfold molar excess of unlabeled −104 fragment was used as a control competitor (Fig. 5A,B, lane 6). This concentration was previously determined to compete significantly for the binding of the labeled −104 probe (data not shown). The various competition reactions were then electrophoresed on a single gel to evaluate and compare the efficiency of each competitor (Fig. 5C). From this competition binding experiment, it is apparent that the PI oligomer had only a minimal effect at best as a competitor when compared with the non-overlapping PII oligomer. A hundredfold molar excess of PII competed approximately threefold better than a 500-fold molar excess of the PI oligomer. The failure of the PI oligomer to significantly compete with the probe in the binding reaction is in accord with the results of Harada et al. [1990]. They showed no detectable amount of the interferon regulatory factors, IRF-1 and IRF-2, at the RNA level in EC cells. Neither of the synthetic DNAs, however, competed as well as a tenfold molar excess of either the −104 fragment (Fig. 5C, lane 10) or the IRE fragment (data not shown). Competition reactions identical to that presented in Figure 5 were performed with extracts from induced F9 cells prepared at another time and the same results were observed.

Comparison of Nuclear Extracts From Induced L929 and F9 Cells

The L929 cell extract results presented in Figure 3 indicated that the 4xHex fragment competed efficiently for binding, yet the same sequence, when used as an annealed oligonucleotide (PI) showed only minimal competition when induced F9 extracts were analyzed (Fig. 5). To rule out artifactual differences due to the source of competitor DNA, induced L929 (Fig. 6, lanes 1–5) and F9 (Fig. 6, lanes 6–10) cell extracts were compared in a competition binding experiment using unlabeled PI and PII oligos as competitor DNAs. Induced L929 competition analysis confirmed that the 4xHex sequence, either as a fragment or oligo (PI) competed initially for the lower band and then at 500-fold molar excess for the upper band. This molar excess, however, was much greater than that indicated in Figure 3. Interestingly, the autoradiogram revealed that the PII oligo competed minimally for both L929 shifted bands. In contrast to the L929 results, the PII oligo competed more efficiently than the PI oligo when induced

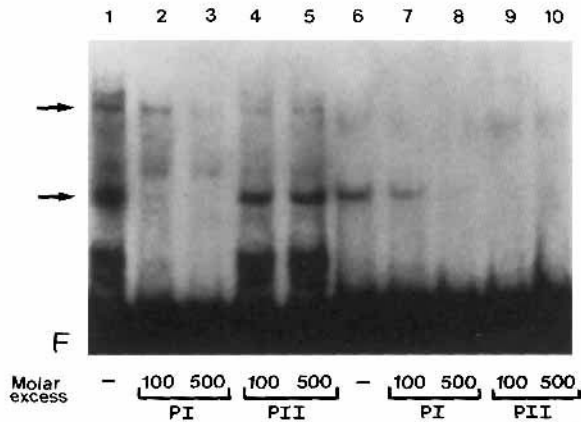

Fig. 6. Comparison of nuclear extracts from induced L929 cells and F9 cells in a competition binding analysis. Nuclear extracts from induced L929 cells (**lanes 1–5**) or F9 cells (**lanes 6–10**) were incubated in the absence (lanes 1, 6) or in the presence of the indicated molar excesses of the PI (lanes 2, 3, 7, 8) or PII (lanes 4, 5, 9, 10) oligonucleotides and 1 ng of the −104 probe. Shifted bands are indicated by the arrows and F is free probe.

F9 extracts were examined (Fig. 6, lanes 7–10). While the gel shift analysis initially showed the induced F9 shifted band and the faster moving induced L929 shifted band to similarly alter the probe mobility, the competition results show that the DNA/protein complexes which the bands represent are not identical. While the PII oligomer hardly competed in the L929 binding reaction, it competed extremely well when F9 extracts are examined in parallel. The PI competitions (Fig. 6, lanes 2, 3, 7, 8) also indicate that the L929 and F9 proteins complexed with the probe DNA exhibit differential affinities for the PRDI region of the IRE.

Analysis of Nuclear Extracts From Differentiated Cells

The data above showed a striking difference in DNA/protein interactions with the −104 fragment and extracts from an IFN producing cell line (L929) and an IFN non-producing EC cell line (F9). Since IFN synthesis has been shown to be developmentally regulated at the levels of biologically active protein and RNA detection, it was of interest to analyze the relationship between undifferentiated and differentiated EC cells at the level of DNA/protein interactions involved in the transcriptional regulation of the β-IFN gene. After extensive retinoic acid (RA) treatment (29 and 42 days) to trigger differentiation into parietal endoderm, the cells were treated with poly-I,C to induce IFN production.

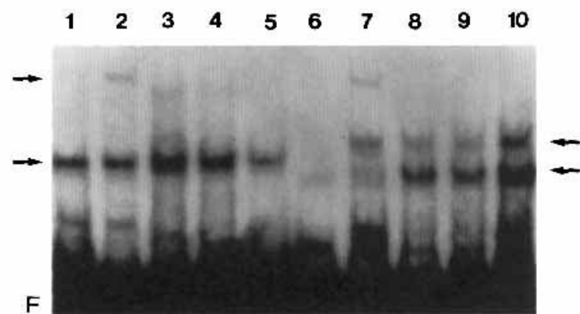

Fig. 7. Gel mobility shift analysis of nuclear extracts from differentiated cells with respect to L929 and F9 nuclear extracts. Extracts from uninduced (**lanes 1, 3, 6, 8**) or induced (**lanes 2, 4, 5, 7, 9, 10**) L929 cells (L1; lanes 1, 2), F9 cells (F9-2: lanes 3–4; F9-1: lane 5), PYS-2 cells (lanes 6, 7) and F9 cells differentiated with RA for 29 days (lanes 8, 9) or 42 days (lane 10) were incubated with 0.5 ng −104 probe and electrophoresed as described in Fig. 3. Shifted bands are indicated by the arrows and F is free probe.

After a 6 h induction period, nuclear extracts were prepared and the cell supernatants were analyzed for antiviral activity (14 and 11 units/ml for 29 and 42 days, respectively, of RA treatment). A gel mobility shift analysis was used to examine the nuclear extracts for DNA/protein interactions with the −104 probe. The results are presented in Figure 7 and show that differences in DNA/protein interactions are apparent upon differentiation of the EC cells (lanes 8–10). Nuclear extracts from the differentiated parietal yolk sac endoderm cell line, PYS-2, were analyzed concomitantly and show a similar pattern of altered probe mobility (Fig. 7, lanes 6, 7). Although the mobility shift pattern differs between the undifferentiated (Fig. 7, lanes 3–5) and the RA-differentiated F9 cells (Fig. 7, lanes 8–10), there appears to be little difference between the uninduced (lane 8) and induced (lanes 9, 10) RA-differentiated F9 extracts. Since only the induced cells produced IFN, the results suggest a more complex DNA/protein interaction than what was detected in this experiment. This interpretation would predict that the differentiated and undifferentiated shifted bands behave differently when subjected to a competition reaction using the PI and PII synthetic DNAs or other competitor DNA fragments as described above.

DISCUSSION

It has been well documented that the multipotential stem cells of a *teratocarcinoma*, the embryonal carcinoma (EC) cells, provide a good

model system for studying differentiation events similar to those in the early stages of mammalian embryogenesis. Previous work by our laboratory demonstrated that the expression of biologically active IFN is developmentally regulated in the teratocarcinoma cell system [Burke et al., 1978]. Further analysis of the molecular mechanism responsible for this regulation showed that the undifferentiated EC cell does not accumulate β-IFN RNA in response to the synthetic IFN inducing agent poly-I,C [Francis and Lehman, 1989; Haggarty et al., 1988; Harada et al., 1990]. In contrast to the undifferentiated EC cells, the RA-differentiated cells showed both detectable antiviral activity and β-IFN RNA in response to either poly-I,C or virus treatment. These studies suggested that the β-IFN gene is not transcribed to a detectable level in response to inducing agents and that control is exerted at the transcriptional level in undifferentiated EC cells. In the present report, experiments were performed consistent with a transcriptional regulatory mechanism to further analyze the DNA/protein interactions that may be involved in the regulation of β-IFN transcription in undifferentiated EC cells as compared to an IFN-inducible cell line.

Nuclear extracts from uninduced or poly-I,C induced F9 cells and IFN inducible L929 cells were examined by gel mobility shift analyses for the presence of DNA binding proteins specific for the upstream regulatory region of the β-IFN gene. This was done with respect to the previously characterized interferon regulatory element (IRE) and positive and negative regulatory domains [Ohno and Taniguchi, 1983; Zinn et al., 1983; Fujita et al., 1985; Goodbourn et al., 1985, 1986; Goodbourn and Maniatis, 1988]. Our choice of a probe for these studies has taken into account observations reported by several groups [Zinn et al., 1983; Fujita et al., 1985]. Variations in defining the region necessary and sufficient for transcriptional control of the β-IFN gene were observed and attributed to differences between cell culture systems. Since our studies examined yet a third cell system, a region larger than the defined IRE was chosen initially to overcome cell system disparities and secondly to provide the natural upstream architecture which may help stabilize the binding of transcriptional factors present in the nuclear extracts. The plasmid, pBS-104 β-IFN, was engineered to facilitate isolation of a 65 bp fragment from position −104 to −39 upstream of the β-IFN transcriptional start site. This larger region contains several additional putative binding sites for the interferon regulatory factor (IRF) proteins, initially described as interacting with PRDI [Miyamoto et al., 1988; Harada et al., 1989, 1990]. A third positive regulatory domain, PRDIII, was recently identified by Leblanc et al. [1990] upstream of PRDI at position −94 to −78. This domain contains two AAGTGA hexamer sequence motifs for IRF binding and when linked to PRDI and PRDII in a plasmid, behaved as a regulated, virus-inducible enhancer, similar to the entire β-IFN promoter. This data also supports the choice of the larger −104 fragment as a probe to assess F9 and L929 cell nuclear extracts for DNA binding proteins specific for the regulatory region of the β-IFN gene. Competition analyses using small unlabeled DNA fragments which represent the various regulatory domains were then performed to assess the specificity of protein binding to the probe when comparing induced F9 and L929 cell extracts. The results suggested a mechanism for understanding the lack of β-IFN expression in EC cells in response to poly-I,C.

In this report, gel mobility shift analyses were initially performed with nuclear extracts from L929 cells. These cells, as an IFN-inducible cell line, were chosen to establish a binding profile with the −104 fragment as the probe. Analysis of poly-I,C induced L929 extracts demonstrated two shifted bands as compared to a single shifted band from uninduced L929 extracts (Fig. 2). These results suggested a difference in DNA/protein complex formation as a result of the induction procedure as expected from previous reports using probes of different sizes. The bands observed with induced extracts represented two specific interactions with the −104 probe as confirmed by competition binding experiments using either the entire probe sequence or smaller portions of it (Fig. 3, 6). While the PII oligomer showed virtually no competition of either complex, a sequence as small as the hexamer binding motif of PRDI (AAGTGA) appeared to be responsible for both binding events. Further analysis using antibodies to specifically block complex formation of previously identified transcription factors such as IRF-1, IRF-2, or NF-κB should help identify L929 proteins complexed to this probe fragment. Additionally, whether these binding events result from different proteins or multimers of proteins has yet to be determined.

These results differed from those presented by Haggarty et al. [1988], who reported one shifted band with L929 extracts. Their extracts, however, were prepared from L929 cells induced with Newcastle Disease Virus and it is unclear at what time post-induction the extracts were prepared. Both the nature and the time of induction may have a significant effect on the binding proteins associated with the DNA. In addition, Haggarty and co-workers used a longer probe consisting of an upstream region from position −238 to position −39. Although providing the natural upstream region, a probe of this length may show differences in protein binding when compared to the shorter probe used in this report.

The results observed with the L929 cell nuclear extracts served as a reference point for examining the F9 cell system. When the induced and mock-induced F9 extracts were examined, only a single shifted band, which co-migrated with the lower band observed with induced L929 cell extracts, was visualized. The specificity of this interaction was subsequently examined by a competition binding experiment. Unlabeled −104 and IRE fragments as well as the PI and PII oligomers, successfully competed in the binding reaction with the −104 probe (Fig. 5 and data not shown). The induced DNA/protein complex appeared not to be identical to the co-migrating complex observed with induced L929 extracts since both PI and PII oligomers competed differently in the binding reactions of these extracts (Fig. 6). Two striking observations were made. First, the PI oligo competed extremely well for both induced L929 complexes and only moderately with the induced F9 complex. Second, the PII oligo competed extremely well with the induced F9 complex and only minimally, at best, for either of the L929 complexes. The competition results with the PII oligo suggest the involvement of NF-κB or an NF-κB–like molecule which binds to the −104 fragment and is present in poly-I,C induced F9 extracts but not in poly-I,C induced L929 cell extracts. On the other hand, in induced L929 extracts, the IRF-1 and IRF-2 molecules or any of the other identified trans-acting factors binding to the PRDI sequence motif [Ohno and Taniguchi, 1983; Zinn et al., 1983; Fujita et al., 1985; Goodbourn et al., 1985, 1986; Goodbourn and Maniatis, 1988] seem to play a stronger role in binding to the −104 fragment. Further analysis needs to be performed to identify the specific factors involved as well as the binding site occupied on the −104 fragment.

The detection of one shifted band with undifferentiated F9 extracts differs from the results of Haggarty et al. [1988] who reported two shifted bands with P19 EC extracts. This discrepancy may be accounted for by cellular differences, the IFN induction procedure, or the time post-induction when the extracts were prepared. In addition no mention was made of an attempt to titrate the amount of carrier DNA (poly-dI:dC) used in the binding reaction. As is indicated in Figure 4, the amount of carrier DNA was critical since two shifted bands were detected in the absence or in the presence of low concentrations of the carrier DNA. The lower band was consistently observed at higher concentrations of carrier DNA and subsequently shown to be a specific interaction with the competition binding analysis (Fig. 5).

Harada et al. [1990] recently reported yet a third result when they examined the developmentally controlled activation of β-IFN at the level of the trans-acting factors, IRF-1 and IRF-2, which bind to the PRDI sequence motif. Their report revealed no bands of altered mobility when nuclear extracts from undifferentiated P19 EC cells were examined by gel mobility shift analysis. This may be as a result of analyzing a different cell line or of their probe consisting only of the PRDI sequence motif, AAGTGA, as a tetrahexamer [Fujita et al., 1988; Harada et al., 1990; Miyamoto et al., 1988]. The utilization of the hexamer repeat, whether in oligo or fragment form, may not truly mimic the binding site as does the −104 fragment. As discussed above, this is supported by the work of Leblanc et al. [1990]. The tetrahexamer, $(AAGTGA)_4$, as a probe may in fact be detrimental to the detection of DNA binding proteins since none were detected in P19 cell nuclear extracts by Harada et al. [1990], while Figures 4–7 of this report clearly indicate a protein/DNA interaction when a fragment from positions −104 to −39 was used as a probe. The results presented in Figures 5 and 6 indicate that the PRDII and to a lesser extent the PRDI sequence motifs are involved in the DNA/protein interaction. In view of our findings showing a DNA/protein complex with the −104 probe and those of Harada et al. [1990], who show neither detectable protein nor mRNA of IRF-1 and -2 in the F9 and P19 EC cell

lines, it is possible that in undifferentiated EC cells NF-κB or an NF-κB–like protein binds to the sequence motif of PRDII. This interaction, however, is insufficient to transcriptionally activate the β-IFN gene. Additionally, it has not been ruled out that the NF-κB–like factor also interacts to a lesser extent (possibly due to lowered affinity) with PRDIII or other PRDI sequence motifs upstream of the strong IRF-1-2 site at position −69 to −76. Further analysis needs to be performed to identify the specific factor(s) interacting with these binding sites since several groups have identified numerous transcriptional activating factors in this region [Ohno and Taniguchi, 1983; Zinn et al., 1983; Fujita et al., 1985; Goodbourn et al., 1985, 1986; Goodbourn and Maniatis, 1988].

Following the RA-triggered differentiation of EC cells, a change was observed in the binding of proteins to the −104 probe. Not only were detectable levels of IFN activity and mRNA observed in response to poly-I,C treatment [Francis and Lehman, 1989; unpublished results], but an altered DNA binding pattern was visualized when extracts from undifferentiated and RA-differentiated cells were compared by gel mobility shift analysis (Fig. 7). The gel shift results are slightly different from those of Haggarty et al. [1988] and Harada et al. [1990] and may be as a result of cell line differences, induction procedure, and/or probe size. Regardless, our results indicated that upon poly-I,C induction of the differentiated F9 cells, the DNA/protein interactions changed. Whether this altered mobility was a result of different proteins binding to the −104 fragment or different modifications to the same protein has yet to be determined.

The results presented in this report show an altered DNA/protein pattern observed in the F9 EC cell model system as it pertains to the developmental control of β-IFN expression. Evidence is reported which supports control at the level of transcriptional activation. In comparison with poly-I,C induced L929 cells which produced IFN and whose nuclear extracts showed two binding events, poly-I,C induced F9 cells produced no detectable IFN activity and nuclear extracts that exhibited only a single binding event with the −104 fragment. These results suggest that a protein/DNA interaction responsible for the transcriptional activation of the β-IFN gene may be absent in undifferentiated F9 cells. In support of this hypothesis is the data from Harada et al. [1990] who show no detectable EC cell protein binding to the PRDI domain and our results showing only moderate competition with the PI oligo and induced F9 extracts (Fig. 5, 6). It appears that a positive transcription factor binding to PRDI is absent in induced undifferentiated EC cells. However, our results using a larger probe and competitions with the PII oligo suggest that there is a protein complexed with the NF-κB sequence motif in induced F9 cells. At this point, it is not known if this protein interaction has suppressor activities as does IRF-2 binding to the PRDI sequence motif [Harada et al., 1989]. Further analysis of this DNA/protein complex in EC cells is needed to identify where the protein contacts the DNA, as well as the nature of the transcriptional factor in terms of its post-translactional modifications. Any or all of this information is likely to be important in the DNA/protein complex formation and to play a role in controlling the developmental expression of β-IFN.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Thomas Friedrich and Ms. Judith Laffin for many useful discussions and critically reading the manuscript, and Ms. Jo Ann D'Annibale for typing the manuscript.

This work was supported by grant CA-41608 from the National Cancer Institute.

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