Repression of Virus-Induced Interferon A Promoters by Homeodomain Transcription Factor Ptx1

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Interferon A (IFN-A) genes are differentially expressed after virus induction. The differential expression of individual IFN-A genes is modulated by substitutions in the proximal positive virus responsive element A (VRE-A) of their promoters and by the presence or absence of a distal negative regulatory element (DNRE). The functional feature of the DNRE is to specifically act by repression of VRE-A activity. With the use of the yeast one-hybrid system, we describe here the identification of a specific DNRE-binding protein, the pituitary homeodomain 1 (Ptx1 or Ptx1). Ptx1 is detectable in different cell types that differentially express IFN-A genes, and the endogenous Ptx1 protein binds specifically to the DNRE. Upon virus induction, Ptx1 negatively regulates the transcription of DNRE-containing IFN-A promoters, and the C-terminal region, as well as the homeodomain of the Ptx1 protein, is required for this repression. After virus induction, the expression of the Ptx1 antisense RNA leads to a significant increase of endogenous IFN-A gene transcription and is able to modify the pattern of differential expression of individual IFN-A genes. These studies suggest that Ptx1 contributes to the differential transcriptional strength of the promoters of different IFN-A genes and that these genes may provide new targets for transcriptional regulation by a homeodomain transcription factor.

The multifunctional secreted interferon (IFN) proteins mediate antiviral defense, immune and cell growth regulation. After virus induction, type I IFN (IFN-A and IFN-B) genes are expressed in a large variety of human and murine cells. The IFN-B gene and the individual subtypes of IFN-A genes are transcribed at various levels depending on cell type or virus inducers, reflecting differences in the transcriptional activity of the corresponding gene promoter in a particular cell type (10, 18, 19). Virus-responsive element B (VRE-B) of the human IFN-B promoter mediates both induction and repression. The positive control of VRE-B depends on many activators, including NF-κB, ATF-2 (c-jun), IRF-3, IRF-7, and HMG I(Y). In association with the transcriptional coactivator CBP (p300), these factors play an essential role in the assembly of a higher-order transcription enhancer complex named the enhancosome (12, 22, 36, 40, 42). The negative control of VRE-B is also the result of different repressors. IRF-2 is able to antagonize activators of the IRF family by competing for their binding (9). NFκB represses the activation due to NF-κB (23), and PRDI-BF1 is a postinduction repressor of the gene (26).

IFN-B and IFN-A genes are transcriptionally activated and repressed through either common or specific mechanisms. IRF-binding sites are conserved in both the VRE-A and VRE-B regions, but binding sites for the factors regulating IFN-B gene expression were not found within VRE-A. Furthermore, even if IFN-A genes are structurally related, differences in the expression of the individual subtypes of the multigenic IFN-A gene family after virus induction are observed. Most attention has been paid to comparisons of some of the different murine IFN-A promoters (5, 7, 25). The murine IFN-A11 gene is poorly expressed upon Newcastle disease virus (NDV) induction, whereas the IFN-A4 gene is strongly inducible. The lack of transcriptional activity of the IFN-A11 promoter could be related in part to substitutions in IRF-binding sites which are present in VRE-A of the IFN-A4 promoter. Recent data suggest that IRF-3 and IRF-7 are also involved in the transcription of the murine IFN-A genes (3, 11, 20, 28, 42).

Whereas a large number of repressors binding to the IFN-B promoter elements have been identified, repression of the IFN-A genes is not well characterized. In addition to substitutions in the proximal VRE-A, the repression of the IFN-A11 gene after virus induction is due to the presence of a distal negative regulatory element (DNRE) of 20 bp, which is delimited upstream of VRE-A (17, 27). This element exerts an inhibitory effect on proximal VRE-A promoters after virus induction, whatever its orientation or position, and is therefore considered a silencer. On the other hand, the DNRE on its own has no effect on VRE-B promoter after virus induction or any constitutive repressive effect on heterologous promoters. Therefore, the functional feature of the particular silencer DNRE is that its silencing activity is strictly dependent on the presence of a functional VRE-A and that it does not function as a general negative regulator. Similar DNREs are present in some IFN-A promoters, and the presence or the absence of DNRE may contribute to the differential expression of the IFN-A genes after virus induction. Furthermore, a DNRE (4DNRE) is also present in the highly inducible IFN-A4 promoter but a central antisilencer region located between the silencer and the VRE-A4 overrides the silencer activity. Two DNRE-binding factors have been observed before virus induc-
tion of murine L929 cells and human HeLa S3 cells and are still maintained even following induction. One of these factors corresponds to the HMG I(Y) protein but does not modulate the binding to DNRE of a second, uncharacterized factor related to the silencer activity (17).

In this study we have used the yeast one-hybrid system to clone a CDNA encoding the human homologue of the mouse bicoil-related putative homeobox 1 (Ptx1 or Ptx1l) that specifically recognizes the DNRE. The DNRE and the Ptx1-binding element are able to repress to the same extent the virus-induced transcription of a theoretical level of VRE-A promoters. We have shown that the Ptx1 gene is constitutively transcribed in cell types that differentially express IFN-A genes after virus induction and that the Ptx1 protein specifically binds the DNRE. Upon virus induction, overexpression of Ptx1 negatively regulates the IFN-A promoters containing the DNRE, and the C-terminal region, as well as the homeodomain of the protein, is required for the trans repression. The central antisilencer region in the highly inducible IFN-A4 promoter overrepresses the repressive activity of Ptx1. Ptx1 antisense RNA experiments showed that endogenous IFN-A expression is quantitatively increased and the pattern of differential gene expression is qualitatively influenced. These data suggest that Ptx1 may exert a modulation on the differential transcriptional strength of the promoters of different IFN-A genes.

MATERIALS AND METHODS

Cloning with the use of the yeast one-hybrid system. The Saccharomyces cerevisiae strains used in this study were all derived from strain YPM954 (MATa ade2 his3 leu2 lys2 trp1 ura3 gal4 gal80::kanR). The double-stranded motifs containing three oriented copies of the wild-type DNRE element from the IFN-A11 promotor, i.e., 5’-cggATTTAAGTAGGATTTAAGTcggATTTAAGTAGGATTTAAAGTcgg-3’ and mutated-DNRE motifs DM1 (5’-cggATTTAAGTAGGATTTAAGTcggATTTAAGTAGGATTTAAAGTcgg-3’), and mutated-DNRE motifs DM2 (5’-cggATTTAAGTAGGATTTAAGTcggATTTAAGTAGGATTTAAAGTcgg-3’), and DM2 (5’-cggATTTAAGTAGGATTTAAGTcggATTTAAGTAGGATTTAAAGTcgg-3’), were made by PCR using single oligonucleotides from E. coli as templates and different oligonucleotides containing their complete open reading frame or cDNAs containing deleted or mutated forms of Ptx1 subcloned in expression plasmids were used. The PTX1 vector (containing the full-length Ptx1 cDNA in reverse orientation) were used. All constructions were checked by nucleotide sequencing on double-stranded DNA template. The resulting plasmids were linearized with Sac3I site. All constructions were described previously (17). In vitro transcription and translation. The proteins were translated with the TNT coupled transcription and translation kit from Promega as specified by the manufacturer. Plasmids pRC-CMV-PTX1 and pRC-CMV-Ptx1 were used for translation of human Ptx1 protein and murine Ptx1 proteins, respectively.

EMSA. Electrophoretic mobility shift assays (EMSA) were performed as described previously (17). In vitro-translated reticulocyte lysates were preincubated with 32P-labeled (300 Ci/mmol) in the presence of the 20 mM salmon sperm DNA for 10 min on ice. The mixture was then added to the binding buffer containing 10 fmoles of [32P]-end-labeled probe (50,000 cpm, 0.1 ng) either with or without competitor oligonucleotides in a final volume of 20 µl, and the incubation was carried out for a further 30 min at room temperature. Complexes were resolved by electrophoresis on prerun 5% polyacrylamide gels. After being dried, the gels were autoradiographed overnight. The following chemically synthesized double-stranded oligonucleotides were used as probes: DM1 (5’-AGTGGCTAGGCTCTGTGCTTTCCTG3’-3’), DM2 (5’-AGTGGCTAGGCTCTGTGCTTTCCTG3’-3’), DM3 (5’-GATGACACGTCCTCGTAGGGCTGCAC3’-3’), and DM4 (5’-CAGCTAATTTAAAGTcggATTTACAGCTACTGTAAAGTcgt3’-3’). In vitro transcription and translation of human PTX1 and murine Ptx1 proteins, respectively.

Results and Discussion

The repression of murine L929 cells and human HeLa S3 cells and are still maintained even following induction. One of these factors corresponds to the HMG I(Y) protein but does not modulate the binding to DNRE of a second, uncharacterized factor related to the silencer activity (17).

In this study we have used the yeast one-hybrid system to clone a CDNA encoding the human homologue of the mouse bicoil-related putative homeobox 1 (Ptx1 or Ptx1l) that specifically recognizes the DNRE. The DNRE and the Ptx1-binding element are able to repress to the same extent the virus-induced transcription of a theoretical level of VRE-A promoters. We have shown that the Ptx1 gene is constitutively transcribed in cell types that differentially express IFN-A genes after virus induction and that the Ptx1 protein specifically binds the DNRE. Upon virus induction, overexpression of Ptx1 negatively regulates the IFN-A promoters containing the DNRE, and the C-terminal region, as well as the homeodomain of the protein, is required for the trans repression. The central antisilencer region in the highly inducible IFN-A4 promoter overrepresses the repressive activity of Ptx1. Ptx1 antisense RNA experiments showed that endogenous IFN-A expression is quantitatively increased and the pattern of differential gene expression is qualitatively influenced. These data suggest that Ptx1 may exert a modulation on the differential transcriptional strength of the promoters of different IFN-A genes.
RESULTS

We have previously identified a silencer element (DNRE) within the murine IFN-A11 promoter which is responsible for the distal repression of IFN-A11 gene after NDV induction in both murine L929 and human HeLa S3 cell lines (17, 27). The similarity of the results obtained with the two cell lines suggests that the factor(s) involved in this repression may be present in both murine and human cell lines. Furthermore, the isolated DNRE of the IFN-A11 promoter or similar elements of DNRE found in other IFN-A promoters are able to reduce the inducibility of these different IFN-A promoters. These results suggest that DNRE may play a general role in the differential transcriptional strength of the IFN-A gene promoters. To confirm the functional role of DNRE, we previously introduced a series of mutations within a DNA-protein interaction site of the IFN-A11 promoter established by DNase I footprinting. For example, while DM1 and DM3 mutations were shown to maintain the negative effect of DNRE after virus induction, the DM2 mutant caused the loss of repression of the promoter (17) (see also Fig. 2).

Cloning of the PTX1 gene by the yeast one-hybrid system.
To identify the gene(s) encoding the factor(s) that could recognize DNRE, the multimerized DNRE element (three copies) was used in a yeast one-hybrid system (Fig. 1A and B), similar to that previously described (4). For the cloning of DNA-binding proteins, a cDNA-Gal4 fusion library from HeLa S3 RNA was used. The first screening yielded different families of positive cDNA-Gal4 fusion clones classified by sequence analysis. Different mutated DNRE were used in the second screening. The mutated plasmids were used to transform strains harboring, integrated in the genome, a HIS3 gene placed downstream of either the DM1, DM2, or DM3 mutant (three copies [Fig. 1A]) of the DNRE motif (YMDM1, YMDM2, and YMDM3). As shown in Fig. 1C, these transformations allowed us to isolate one plasmid expressing a Gal4 fusion protein which activated the transcription of the HIS3 gene placed downstream of the DNRE or the DM1 or DM3 mutants but not the DM2 mutant. The sequence of this 1-kb cDNA indicated a partial open reading frame of 678 bp. Sequence comparison with databases indicated that it is a human protein that contains a homeodomain (HD) which is identical to PTX1 (30). Another plasmid was isolated by this screening. This plasmid expressed the neighbour of tid (Not) 56 protein (GenBank accession no. Y09022), whose function remains unknown. This Gal4 fusion protein activated the transcription of the HIS3 gene placed downstream of the DNRE or the DM3 mutant but not the DM1 or DM2 mutants and was not further investigated.

Homeotic genes encode transcriptional factors involved in the positive and negative regulation of target genes during development. These genes contain a highly conserved sequence of 180 bp, the homeobox, which encodes a 60-amino-acid polypeptide, the HD, which represents the DNA-binding domain of these factors. The partial cDNA cloned by the yeast one-hybrid system contains the complete HD of the protein between nucleotides 264 and 444. The amino acid sequence of the human transcription factor showed 96% homology to that of murine Ptx1 (13). Ptx1 was first described as a transcriptional activator binding the CE3 element of the pituitary pro-opiomelanocortin (POMC) gene promoter (13). It activates the transcription of other pituitary genes (37). Studies with Ptx1-deficient mice indicate that hindlimb patterning and man-
such as CE3M and DM2 had a poor effect. Human PTX1 protein gave similar results (data not shown). In conclusion, Ptx1 binds specifically to DNRE both in vivo and in vitro and its binding properties are consistent with the activities of the DNRE mutants. The same specific binding of Ptx1 was detected using the 4DNRE of the IFN-A4 promoter (Fig. 3C and data not shown), suggesting that different DNREs may be the bicoid-binding site for this factor. Essentially identical results were obtained for the CE3 sequence as a probe but with a higher affinity than for the DNRE. CE3M had no effect. These results, showing that the Ptx1 protein binds specifically to the DNRE and CE3, are in agreement with the ability of these elements to repress the virus-induced transcriptional level of IFN-A promoters. Then the pattern of PTX1 or Ptx1 gene expression was assessed in cell types that express IFN-A genes. PTX1 or Ptx1 genes are constitutively transcribed in cell types that are able to differentially express IFN-A genes. Ptx1 is expressed in adult anterior pituitary cells and during pituitary development (13, 15). However, the expression of the murine Ptx1 or the human PTX1 genes is not restricted to the pituitary cells; these genes are also expressed during embryogenesis and in adult tissues in derivatives of posterior lateral plate mesoderm (13, 14, 30). To date, transcription of murine Ptx1 and human PTX1 genes has not been described in cell

![Diagram of DNRE and DNRE mutants](image-url)
FIG. 2. Repression of the virus-induced transcriptional level of VRE-A11 and VRE-A4 by the Ptx1 element CE3. (A) Homologies between the core consensus recognition DNA sequence for the binding of Ptx or bicoid proteins and CE3 in the DNRE sequences. The nucleotide substitutions are underlined, and the homologies are boxed.

(B) Effect of CE3 in the IFN-A11- and IFN-A4-proximal promoters. Plasmid constructs with the CAT gene under the control of the indicated promoter fragments were tested by measuring the CAT activity. L929 and HeLa S3 cells were transfected and mock induced (open bars) or NDV induced (solid bars) as described in Materials and Methods. Since the data are pooled from several experiments, they are presented in arbitrary units of CAT activity. CAT activities for each plasmid are the means and standard errors (SE) for at least five separate transfections with at least two separate plasmids. Error bars indicate SEs. Virus inducibility is the ratio of the NDV-induced activity over the mock-induced activity.
types that are shown to differentially express IFN-A genes after virus induction, such as human PBL (10). In this study, the total RNAs of different cell types were isolated for detection of PTX1 or Ptx1 mRNA by RT-PCR. RT-PCR primers (exon 2 and exon 3 primers) were designed to detect both murine Ptx1 and human PTX1 mRNA. These primers were also designed to exclude amplification of other members of the Ptx family. RT-PCR products were cloned and analyzed by DNA sequencing. RT-PCR analysis of RNA from uninduced human PBL, monocytes, and lymphocytes and subsequent sequence analysis of RT-PCR products (data not shown) showed that Ptx1 mRNAs are constitutively expressed in these cells (Fig. 4A, lanes 1 to 4). PTX1 transcripts were detected in the starting population of PBL in three experiments performed with cells from different donors (data not shown). PTX1 gene expression could also be detected by RT-PCR in different cell lines such as epithelial HeLa S3 cells, from which we have cloned PTX1 in this study, promyelocytic HL60 cells, and monoblastic U937 cells, but not in myeloblastic KG1 cells (lanes 5 to 8). No amplification was observed in the absence of the reverse transcription steps (data not shown). Our previous results suggest that the same type of murine IFN-A promoter regulation is present in AtT-20, HeLa S3, and, to a lesser extent, L929 cells. The binding was increased using extracts from L929 cells transiently transfected with Ptx1 expression vector (L929 Ptx1-S). We also generated Ptx1 knockdown cell lines by stably

FIG. 4. Human PTX1 and murine Ptx1 expression in cell types expressing IFN-A genes. Expression of PTX1 and Ptx1 genes in different cell types and cell lines was monitored by RT-PCR using primers designed to exclude amplification of other members of the Ptx family (see Materials and Methods). RT-PCR products were also cloned and analyzed by DNA sequencing (data not shown). (A) PTX1 gene expression. Total RNA was extracted from different human cell types as well as cell lines and was monitored by RT-PCR. As a control, expression of GAPDH mRNA is shown in the lower panel. (B) Ptx1 gene expression. Total RNA were extracted from NDV-induced L929 cells at 0, 8, and 18 h postinduction. AT-20 cell line was used as a control for the expression of the Ptx1 gene. Expression of Ptx1 and GAPDH mRNA was monitored by RT-PCR. (C) Endogenous Ptx1-binding activity to DNRE in different cell lines. Nuclear extracts from different cell lines were used for EMSA in the presence of related Drosophila bicoid target site (Db), CE3 and DNRE probes (lanes 1 to 14). The AT-20 cell line expressing the Ptx1 protein was used as a control. The binding activity was monitored using nuclear extracts from HeLa S3 cells, L929 wild-type cells, a L929 control clone (L929 Ctl), L929 cells transiently transfected with the Ptx1 sense expression vector (L929 Ptx1-S), and L929 clones stably transfected with the Ptx1 antisense RNA expression vector (L929 Ptx1-AS). The quality of nuclear extracts was tested by EMSA for YY1-binding activity (lanes 15 to 20). (D) Specific binding of Ptx1 to the wild-type DNRE probe. Nuclear extracts from AT-20 and HeLa S3 cell lines were incubated with CE3 or DNRE probes and a 50-fold molar excess of unlabeled CE3, CE3M, DNRE, DM1, DM2, and DM3. (E) The DNRE binding of recombinant Ptx1 (lanes 1 to 3) and nuclear protein of L929 cells (lanes 4 to 6) was supershifted by the addition of an antisem against maltose-binding protein-Ptx1 (anti-Ptx1) but not by the addition of preimmune serum.
transfecting a Ptx1 antisense RNA expression vector in L929 cells (L929 Ptx1-AS). Three independent neomycin-resistant clones expressing Ptx1 antisense RNA were analyzed. A clone stably transfected with the same vector without the Ptx1 cDNA was chosen as a control (L929 Ctl). In the pool of three Ptx1 antisense clones, CE3- and DNRE-binding activities were almost undetectable, whereas another transcription factor such as YY1 was not significantly affected (lanes 15 to 20). The specificity of the DNA-binding activities of nuclear extracts from AtT-20 and HeLa S3 cell lines was also observed. Using CE3 as a probe (Fig. 4D, lanes 1 to 3), the binding activity present in AtT-20 cells was competed by CE3 and DNRE used as cold competitors. Using CE3 and DNRE as probes with nuclear extracts from AtT-20 and HeLa S3 cell lines and with human PTX1 overexpression (data not shown), the DNRE-binding activities of nuclear extracts from AtT-20 and HeLa S3 cell lines, Ptx1 overexpression led to a significant decrease (more than 80%) in the transcriptional activity of this promoter (solid bars). Titration of the Ptx1 plasmid suggested that the level of native IFN-A11 promoter repression after virus induction was maximal. Similar results were observed when using human PTX1 overexpression (data not shown).

After virus induction, Ptx1 overexpression led to a significant (16-fold) repression of constructs containing the DNRE upstream of the IFN-A11 proximal promoter (Fig. 5B, lane 2) and Ptx1 repressed neither the promoter lacking the DNRE nor the promoter containing the mutated DNRE, DM2 (lanes 1 and 3). A repression was also observed with constructs containing three copies of the DNRE, the 4DNRE, or the DNRE (20-, 8-, and 10-fold repression, respectively) upstream of the proximal promoter of the IFN-A4 gene, which is strongly inducible (lanes 5 to 7). No effect was observed in the absence of the DNRE or the 4DNRE or in the presence of DM2 (lanes 4 and 8). Similar results were observed using human PTX1 overexpression (data not shown). In addition, viral Rous sarcoma virus, cytomegalovirus, and thymidine kinase promoters, as well as the elongation factor promoter, were poorly or not sensitive to Ptx1 overexpression (data not shown). Thus, these results suggest that after virus induction, the Ptx1 protein specifically represses the transcription of the IFN-A11 and IFN-A4 proximal promoters in the presence of DNRE. The fact that the intact IFN-A4 gene promoter remains highly inducible upon virus induction has been previously explained by the presence of a third element in the IFN-A4 promoter. This element is a central region located between the distal 4DNRE and the proximal VRE-A of the IFN-A4 promoter.
and is able to overcome the DNRE silencer activity. Therefore, this element, named 4D, has been considered an antisilencer (17). Our results show that the repressing activity of Ptx1 was abolished in the context of the native IFN-A4 promoter (Fig. 5C). In contrast, in the absence of the central antisilencer region 4D, the repressing effect of Ptx1 was observed. Thus, Ptx1 inhibits the virus-induced transcriptional activity of the IFN-A11 promoter through DNRE but not the IFN-A4 promoter containing both the distal 4DNRE and the central antisilencer region 4D. Thus, DNRE and Ptx1 appear to function as a context-dependent repressors.

The C-terminal region and the HD of Ptx1 are required for trans repression. To characterize the transcriptional repressive domain(s) of Ptx1, we used truncated forms of Ptx1 (38, 39) lacking either the N-terminal or the C-terminal regions but containing the HD (Fig. 6A). The bicoid-related HD is characterized by a lysine residue at position 50 of the HD. This lysine residue determines the DNA-binding specificity, and its
FIG. 6. Trans repression of IFN-A11 promoter activity by expression constructs of Ptx1. (A) Ptx1 expression constructs. The HD of Ptx1 is shown. The N-terminal truncation in mutant Ptx1 delta NH2, the C-terminal in mutant Ptx1 delta COOH, and the lysine residue changed to alanine in Ptx1 mutant K50A are shown. (B) L929 and HeLa S3 cells were cotransfected with the IFN-A11/luciferase reporter construct (-457A11wt-Luc) and with empty expression vector or vector encoding Ptx1, Ptx1 delta NH2, or Ptx1 delta COOH, as indicated. Luciferase expression in cotransfected cells is expressed relative to the induced activity of -457A11wt-Luc alone, which was set at 100%. Assay conditions were as described in the legend to Fig. 2B, except that the luciferase activity was determined. (C) L929 cells were cotransfected with the IFN-A11/luciferase reporter construct (-457A11wt-Luc) and with empty expression vector, vector encoding Ptx1, or vector encoding Ptx1 mutant K50A, as indicated. Assay conditions were as described in the legend to Fig. 2B. Luciferase activity after virus induction is reported as fold repression relative to the -457A11wt-Luc construct, which was set at 1.
mutagenesis (Fig. 6A) abrogates DNA binding (32, 41). The expression level and nuclear localization of Ptx1 mutant proteins have been assessed previously (38, 39). The N-terminally truncated form of Ptx1 had the same effect as the full-length Ptx1, but the C-terminally truncated and HD mutated forms of Ptx1 were unable to repress the IFN-A11 promoter after virus induction (Fig. 6B and C). These results suggest that the C-terminal region, as well as the HD of Ptx1, is required for the trans repression.

FIG. 7. Endogenous Ptx1 participates in repression of virus-induced IFN-A gene expression. (A) Endogenous Ptx1 factor is essential for promoter-specific repression. L929 wild-type cells or L929 control clone (L929 Ctl) or L929 clones stably transfected with Ptx1 antisense RNA expression vector (L929 Ptx1-AS) were cotransfected with the IFN-A11/luciferase reporter construct (-457A11wt-Luc) and with empty expression vector or vector encoding Ptx1 sense (Ptx1-S) or Ptx1 delta COOH, as indicated. Luciferase expression in cotransfected L929 wild-type cells is expressed relative to the induced activity of -457A11wt-Luc alone, which was set at 100%. Assay conditions were as described in the legend to Fig. 6B. L929 or L929 Ptx1-AS cells were also transfected with various constructs corresponding to the IFN-A11, IFN-A4, and IFN-B promoters. Assay conditions were as described in the legend to Fig. 6B. Luciferase activity after virus induction is reported as fold repression relative to the L929 wild-type, which was set at 1. (B) Ptx1 antisense RNA experiments lead to an increase of endogenous IFN-A expression. Expression and quantification of IFN-A and IFN-B genes after virus induction in L929 and L929 Ptx1-AS cell lines (0, 4, 6, 8, 10, and 12 h postinduction) were monitored by using RT-PCR consensus conserved primers for IFN-A mRNA or specific primers for IFN-B mRNA. The level of IFN mRNA was quantified (8 h postinduction) by using serial dilution RT-PCR. As a control, expression of GAPDH mRNA is shown in the lower panels.
Endogenous Ptx1 participates in the repression of virus-induced IFN-A gene expression. The importance of endogenous Ptx1 in IFN-A promoter activity was tested by stably transfecting a Ptx1 antisense RNA expression vector in L929 cells. Ptx1 antisense expression led to a significant decrease in Ptx1 DNA-binding activity without affecting other DNA-binding proteins (Fig. 4C). To test the contribution of Ptx1 to IFN-A11 repression, the native IFN-A11 promoter containing the DNRE was transfected into Ptx1 antisense clones (Fig. 7A, upper panel). The use of Ptx1 knockdown cell lines led to a significant increase in IFN-A11 promoter activity after virus induction, whereas no effect was observed in the absence of virus induction. When the Ptx1 sense expression vector was cotransfected into Ptx1 knockdown cell lines together with a native IFN-A11 promoter, the enhanced activity was abolished. Cotransfection of the C-terminally truncated form of Ptx1 has no effect. Deletion of the DNRE-binding site abolished the effect of the Ptx1 antisense RNA (Fig. 7A, lower panel). Furthermore, when the IFN-B promoter or the native IFN-A4 promoter containing the antisilencer region 4D was used, the effect of Ptx1 antisense RNA was not observed.

To further characterize the role of Ptx1 in IFN-A gene repression after virus induction, we quantitatively and qualitatively compared the endogenous IFN-A expression in L929 wild-type and L929 Ptx1 knockdown cells. In these cell lines, although no biologically active IFN protein was observed in culture media before virus induction, a different IFN activity was detected after virus induction. Indeed, at 18 h postinduction, approximately 6,400 and 102,400 IU/ml was titrated for IFN activity of L929 wild-type and L929 Ptx1 knockdown cells, respectively. The difference in IFN protein production between Ptx1 knockdown cells and wild-type cells was 16-fold. Total RNAs of both cell types were isolated before and after virus induction of IFN mRNA for quantification by RT-PCR using consensus conserved primers for IFN-A mRNA or specific primers for IFN-B mRNA. First, L929 wild-type cells expressed much lower levels of IFN-A mRNA in response to virus induction than did L929 Ptx1-AS cells (Fig. 7B, lanes 1 to 12). In both cell lines, IFN-A mRNA were undetectable in the absence of virus induction (lanes 1 and 7). At 8 h postinduction, the level of IFN mRNA was quantified by using serial dilution RT-PCR as previously described (20). IFN-B mRNA and IFN-A mRNA were both undetectable in the absence of virus induction (lanes 13 and 19). Although IFN-B gene expression level was identical in both cell lines after virus induction, IFN-A gene expression was significantly increased in L929 Ptx1 knockdown cells (lanes 14 to 18 and 20 to 24). Indeed, at 8 h postinduction, IFN-A mRNA from wild-type cells was poorly detected following fivefold dilution of cDNA (lane 15). In contrast, IFN-A mRNA from Ptx1 knockdown cells was clearly detected following 25-fold dilution (lane 22). RT-PCR products were quantified by PhosphorImager analysis, and the difference in IFN-A mRNA induction between Ptx1 knockdown cells and wild-type cells was found to be 22.8-fold. These results are in agreement with our previous results of biologically active IFN protein production. Thus, induction of IFN-A gene expression, but not of IFN-B gene expression, was significantly increased after virus induction in Ptx1 knockdown cells.

To distinguish the subtypes of IFN-A gene expression from virus-induced wild-type and Ptx1 knockdown cells, cDNA were cloned and 83 randomly selected clones were analyzed by DNA sequencing. As expected, IFN-A gene expression by wild-type cells displayed a mixture of distinct subtypes (Table 1). IFN-A4 was the most abundant species detected. No cDNA clones for IFN-A11 was detected. In contrast, the IFN-A11 subtype was detected in virus-induced Ptx1 knockdown cells. Strikingly, IFN-A5 but not IFN-A4 was the most abundant species detected, suggesting that, as with the IFN-A11 gene, Ptx1 is essential for IFN-A5 repression. These results suggest that not only is endogenous IFN-A expression quantitatively affected by Ptx1 but also Ptx1 qualitatively influences the pattern of differential IFN-A gene expression.

**TABLE 1. Representation of IFN-A subtypes in virus-induced L929 wild-type and L929 Ptx1 knockdown cells**

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<tr>
<th>IFN-A subtype</th>
<th>No. of clones with subtype/total no. in:</th>
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<tr>
<td></td>
<td>L929 cells</td>
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<tr>
<td>A2</td>
<td>4/39</td>
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<tr>
<td>A4</td>
<td>30/39</td>
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**DISCUSSION**

The analysis of the distal silencer element DNRE, responsible for the virus-induced transcriptional repression of some IFN-A promoters, led us to clone and study the HD transcription factor, Ptx1. We show here that the promoters of IFN-A genes constitute targets for Ptx1 and that Ptx1 plays a role in the differential repression of these genes.

**Ptx1, a repressor of virus-induced IFN-A gene expression.** The mechanism by which Ptx1 represses virus-induced IFN-A gene expression seems to be unrelated to other mechanisms previously described for different negative factors in the IFN-A and IFN-B gene promoters. The first factor found to repress IFN-A and IFN-B gene transcription was IRF-2 (9). IRF2 binds the VRE-B of the IFN-B gene and also recognizes the IRF-binding sites present in the VRE-A of the IFN-A genes (1, 2, 9). The role of IRF-2 as a negative regulatory factor in IFN-A and IFN-B gene expression was confirmed by the use of IRF-2-deficient mice (21). IRF-2 is described as a repressor of transcription because of its ability to antagonize activators by competing for the IRF-binding sites. In contrast to IRF-2, Ptx1 is not involved in competition for overlapping activator(s) DNRE-binding site. Furthermore, IRF-2 is not involved in the differential regulation of IFN-A genes whereas Ptx1 is implicated in this type of regulation.

Another repressor factor binding the VRE-B, PRDI-BF1, has been isolated. PRDI-BF1 is a virus-inducible gene and has been considered a postinduction repressor of the IFN-B gene (26). In contrast to PRDI-BF1, Ptx1 is present before and after virus induction.

On the other hand, NRF binds the VRE-B, and expression of the NRF antisense RNA releases the constitutive endogenous IFN-B gene transcription (23). Thus, NRF is a critical component of IFN-B gene silencing prior to viral induction. In the present study, Ptx1 antisense RNA experiments show that IFN-A mRNA was undetectable in the absence of virus induction whereas IFN-A gene expression was significantly increased in L929 Ptx1 knockdown cells after virus induction. In contrast to NRF, Ptx1 is not involved in the constitutive silencing of IFN-A promoters. Thus, our data suggest a novel mechanism by which Ptx1 represses virus-induced IFN gene expression.

With regard to previously reported results concerning the overexpression of Ptx1 which led to activation of the POMC and other pituitary genes (13, 37), the effects of Ptx1 vary with promoter context, and this is the first demonstration that Ptx1...
can repress gene transcription. Ptx1 now joins the class of transcription factors with dual activator-repressor functions. HD transcription factors function by positively or negatively regulating spatial and temporal patterns of gene expression. Some of these transcription factors, depending on their different promoter contexts, can positively or negatively regulate transcription. For example, the HD transcription factor Oct-1, which activates different promoters, is also involved in repression of the human PIT1 gene expression (6).

The present study shows that the HD transcription factor Ptx1 can modulate POMC or other pituitary genes and IFN-A gene expression differently. The opposite functions of Ptx1 factor may be due to the context of pituitary gene and IFN-A gene promoters. Indeed, the activity of Ptx1 as a positive regulator of transcription is synergized by cell-restricted transcription factors to confer pituitary-, lineage-, and promoter-specific expression. Several known transcriptional interaction factors act in synergy with Ptx1: basic helix-loop-helix NeuroD1 for corticotroph-specific transcription of POMC (24), Pit1 to stimulate expression of the prolactin gene (34, 37), SF-1, an orphan nuclear receptor, and Egr-1, an immediate-early response gene, to stimulate the expression of the bLH gene (37–39).

The C-terminal region of Ptx1 is involved in both transcriptional activation and physical interaction with Pit1, SF1, or Egr-1. In the context of the IFN-A gene promoters, the Ptx1 factor acts as a repressor and its effect is observed only after virus induction on the VRE-A-positive activity. Ptx1 could modulate the activity of specific transcription activators involved in the regulation of the IFN-A gene expression by interaction with factors which bind to VRE-A. Different factors may be required for maximal activity of the IFN-A promoter after virus induction. Two factors of the IRF family, IRF-3 and IRF-7, have been characterized (3, 11, 20, 28, 42). Thus, Ptx1 may interact with these specific IRF factors. Furthermore, our results suggest that the transcription-repressive effects of Ptx1 are due to the C-terminal region of the protein. This last region, which has been found to interact with different factors synergizing the activity of pituitary gene promoters, may be also required for protein interactions with other factors such as IFN factors repressing the virus induction of IFN-A gene promoters.

Ptx1 and differential activation and repression of IFN-A genes. IRF-binding sites are not the only cause of the differential IFN-A gene expression. Indeed, the repression of the murine interferon-a gene after virus induction is also due to the negative regulatory element DNRE and the binding of Ptx1. Furthermore, the DNRE of the IFN-A11 promoter or the similar element 4DNRE found in the IFN-A4 promoter is able to bind Ptx1, which reduces the transcriptional activity of proximal VRE-A in both the IFN-A11 and IFN-A4 promoters, but Ptx1 was unable to repress the virus induction of murine IFN-B promoter.

These results demonstrate that Ptx1 functions as a promoter-specific repressor. On the other hand, the fact that the intact IFN-A4 gene promoter remains highly inducible upon virus induction whereas the intact IFN-A11 gene promoter is poorly expressed has been previously explained by the presence of a third element in the IFN-A4 promoter which is absent in the IFN-A11 promoter. This element is a central region located between the distal 4DNRE and the proximal VRE-A of the IFN-A4 promoter and is able to overcome the DNRE silencer activity. Therefore, this element has been considered an antisilencer (17). The present study shows that the central antisilencer element is able to overcome the repressive effect of Ptx1, and this result clearly demonstrates that Ptx1 functions as a context-dependent repressor. On the other hand, endogenous IFN-A expression is quantitatively affected by Ptx1. Moreover, Ptx1 qualitatively influences the pattern of differential IFN-A gene expression. Indeed, the IFN-A11 subtype was detected in virus-induced Ptx1 knockdown cells. In addition, IFN-A5, not IFN-A4, was the most abundant species detected, thus suggesting that, as with the IFN-A11 gene, Ptx1 is essential for IFN-A5 repression. For the IFN-A5 gene, a sequence (−554 to −549) [TATCCT] in the noncoding strand within the promoter is totally homologous to the core consensus recognition DNA sequence for bicoid-related proteins. The participation of this element in the repression of the transcription of the IFN-A5 gene is thus confirmed. In conclusion, the results of this study suggest that DNRE and Ptx1 may exert a more general modulation on the differential transcriptional strength of the promoters of different IFN-A genes. Therefore, depending on the presence or the absence of different binding sites, the modulator effects of factors such as IRF-3 and IRF-7 as activators and Ptx1 as a repressor play a role in the differential expression of the IFN-A genes after virus induction.

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REFERENCES


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