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Characterization of a mouse interferon gene locus II. Differential expression of α-interferon genes

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ABSTRACT

A cluster of four MuIFN-α genes was recently isolated and characterized (1); one of the genes in this cluster had, in the coding region, an internal deletion of 5 amino acids. Bacterial expression plasmids were constructed to examine the effect of this deletion on the antiviral activity of the MuIFN-α_4 peptide and it was found that the α_4 interferon peptide had a 100-fold lower antiviral activity than full length α-interferon proteins when expressed in E.coli.

Three of the four MuIFN-α genes identified were expressed coordinately in L-cells infected with NDV. The relative levels of α_4 mRNA were substantially higher than the levels of the other α mRNAs. Comparison of the 5' end flanking sequences of these four α interferon genes revealed that the promoter sequences of α_1, α_5 and α_6 are more homologous to each other than to the α_4 promoter which also contains a G rich cluster not seen in the other three promoters.

INTRODUCTION

Very little is presently known on how interferons elicit their various biological effects such as antiviral and anti-cellular activities and immune system modulations. To delineate the fine details of the biology of the interferon system and the mechanism of its action, the mouse model offers many advantages when compared to the human one. The genetics of the interferon system can be studied in detail in mice, where a wide variety of inbred and recombinant strains are available; the ability to induce interferon synthesis with a wide range of viral and non-viral inducers on a single genetic background allows to determine the specificity and variation in the expression of murine interferon genes. And finally, the role of the interferon system in viral infection and modulation of cellular responses can be more easily studied in mouse than other models.

We have shown that the murine type 1 interferon locus is localized on mouse chromosome 4 and in our initial study on the molecular characterization of this locus, we isolated a cluster of four α interferon genes in a 28kb long fragment of mouse DNA (1). Detailed analysis of this cluster indicated that three of the four α genes (α_1, α_5 and α_6) show close DNA homology (92-95%) to each other and all of these α genes contained a 950 nucleotide long homologous sequence in their 5' flanking region. The fourth gene (α_4), which has lower nucleotide sequence homology to the other three genes identified, does not have the repeated intergenic sequence associated with its 5' region and contains a 15 nucleotide long in-frame deletion which removes 5 amino acids from the mature protein at positions 103-107.

The possible role of the identified homologous intergenic sequence in the regulation of expression of the MuIFN-α genes is of interest. The role of cis DNA fragments in the expression of viral and cellular genes has been well documented. Enhancer functions have been shown to be independent of orientation of the enhancing element, which may be localized at either end of a gene. To determine whether the presence of the homologous intergenic sequence has any obvious effect on the expression of the α-interferon genes which it is associated with, in the present work we have determined the levels of expression of the four linked α-interferon genes in virus infected L-cells, and examined whether the expression of these genes can be directly related to the presence of the homologous intergenic sequence or to differences in their promoter sequences. Furthermore, we examined the effect of the internal deletion in MuIFN-α_4 on the antiviral activity of the α_4 peptide synthesized from a prokaryotic expression vector in E.coli.

MATERIALS AND METHODS

Nuclease S1 analysis

5' ^{32}P-labeled probes for S1 nuclease analysis were prepared as follows. 10 μg of plasmid DNA were digested with the appropriate restriction enzyme and treated for 60 min. at 65^{o}C with 200 units of bacterial alkaline phosphatase

(Bethesda Research Laboratories) in 100 µl of 50mM Tris-HCl, pH8.5. The dephosphorylated DNA was purified by phenol/chloroform extraction followed by ethanol precipitation, and labeled with 10 units of T4 kinase (Bethesda Research Laboratories) according to Maxam and Gilbert (2). After purification by phenol/chloroform extraction and ethanol precipitation, the labeled DNA was digested with a second restriction enzyme in order to obtain fragments labeled at only one 5' end and electrophoresed through 1.0% low melting point agarose (Bethesda Research Laboratories). The desired DNA fragments were visualized by ethidium bromide staining and purified by melting gel slices at 65°C, followed by phenol/chloroform extraction and ethanol precipitation. The nuclease S1 analysis performed was a modification of the Berk-Sharp (3) technique as described by Weaver and Weissmann (4). RNA and ^{32}P labeled DNA were hybridized at 50°C for 24 hr, and incubated with 300 units of nuclease S1 (Bethesda Research Laboratories) for 15 min. at 37°C. The treated samples were ethanol precipitated and electrophoresed through 5% polyacrylamide, 7M urea gels and exposed to Kodak XAR-5 film for 1-10 days.

Inductions of MuIFN-α's by Newcastle disease virus

Mouse L-cells were induced with the New Jersey strain of Newcastle disease virus (NDV) as follows. Confluent L-cells were infected with NDV in serum-free Dulbecco's modified Eagle's medium (DMEM) with a multiplicity of infection of 10 for 90 min. Then the medium with virus was removed and replaced with DMEM supplemented with 2% heat inactivated fetal calf serum (Δ FCS) and 50 µg/ml gentamicin. At various times after virus removal, the cells were trypsinized and pelleted for RNA preparation.

RNA extraction and Northern analysis

Total cytoplasmic RNA from L-cells was extracted by a modification of the procedure of Favaloro, et al. (5) using vanadyl-ribonucleoside complexes (Bethesda Research Laboratories). RNAs were denatured in 2.2M formaldehyde and analyzed by electrophoresis through 1.1% agarose gels as described by Lehrach,et al. (6), and transferred to nitrocell-

ulose membranes according to Thomas (7). Hybridizations were performed with the MuIFN-α_2 BglII/HincII probe as described (8). Membranes were exposed to Kodak XAR-5 film for 16-24 hrs.

Construction of bacterial expression plasmids

The expression vectors used were provided by Dr. M. Inouye. MuIFN-α coding regions were inserted behind the promoter for the E.coli outer membrane lipoprotein (lpp) gene (9) which is under the control of the lac^{po} region (10). The expression of the interferon genes is regulated by the lac repressor which is encoded on the same plasmids. Recombinant plasmids were transfected into E.coli JA22I and identified by the colony hybridization method of Grunstein and Hogness (11) using the MuIFN-α_2 coding region as a probe.

Assays of interferon in bacterial lysates

Cultures of E.coli JA22I containing the MuIFN-α expression plasmids were grown in LB broth with 100 μg/ml ampicillin (Sigma) to 50 Klett units, and induced by the addition of isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 2mM. The bacteria were harvested at 100 Klett units and centrifuged at 10,000 rpm for 10 min. The bacterial pellet was resuspended in 50mM Tris-HCl (pH8.0), 30mM EDTA and one-tenth volume of 10mg/ml lysozyme was added. The mixture was incubated at 4°C for 30 min. and then subjected to three cycles of freeze-thawing. After centrifugation at 40,000 rpm for 60 min., the supernatant was stored at -70°C. Serial dilutions of the bacterial lysates were made in 3% ΔFCS, DMEM and assayed for inhibition of the cytopathic effect (12) of encephalomyocarditis virus (EMC) on L-cells. The levels of mouse interferon in each sample were quantitated by comparison with international standards of mouse interferon used in each assay. Neutralization titers of goat anti-mouse interferon sera (provided by Dr. E. DeMaeyer) were determined by incubation of serial dilutions of the antisera with 50 units of interferon from the bacterial lysates for 1 hr at 37°C and then assayed as described above.

RESULTS

The effect of deletion on the antiviral activity of the Mu α_4 peptide

To determine whether the 15 bp deletion in the coding region of the MuIFN-α_4 gene had any effect on the antiviral activity of its encoded protein, this gene was cloned into a bacterial expression plasmid behind the lipoprotein promoter (lpp) under the control of the lac^{po} region (13) to give a fusion product containing the mature protein plus a portion of its signal sequence and several amino acids from the bacterial lipoprotein gene (Fig. 1). The MuIFN-α_4 gene shares significant homology with MuIFN-α_2, including the extra amino acid at the carboxyl terminus and a common BglII site upstream of the deletion in α_4. In order to compare the activity of the α_4 protein with its 5 amino acid deletion to a protein of normal length, and to determine that the alteration in biological activity is due to the deletion and not due to other changes in the primary structure of the protein, the deleted region of the α_4 plasmid was replaced with the same region of α_2 at their common BglII site (Fig. 1).

The biological activities of the α_4 interferon peptide and the hybrid were determined on the basis of their antiviral activities. The antiviral activity of the bacterial lysates (prepared as described in Materials and Methods) was assayed on mouse L-cells challenged with EMC virus; while the hybrid α_4/α_2 fusion protein yielded 1600 units/ml of bacterial culture, the deleted gene, α_4, produced only 16 units/ml (Table I). Interferon peptides encoded by similar expression plasmids of MuIFN-α_1, -α_5 and -α_6 have levels of antiviral activity comparable to that of the hybrid fusion α_4/α_2 protein (Kelley and Pitha, unpublished observations). Both bacterial fusion proteins were completely neutralized by goat antiserum raised against mouse interferon (provided by Dr. E. DeMaeyer). While a 1:2400 dilution of this serum neutralized 50 units of natural mouse IFN (a mixture of α and β), a 1:1200 or 1:600 dilution of this serum was needed to neutralize 50 units the α_4 fusion protein or α_4/α_2 hybrid protein, respectively (Table I).

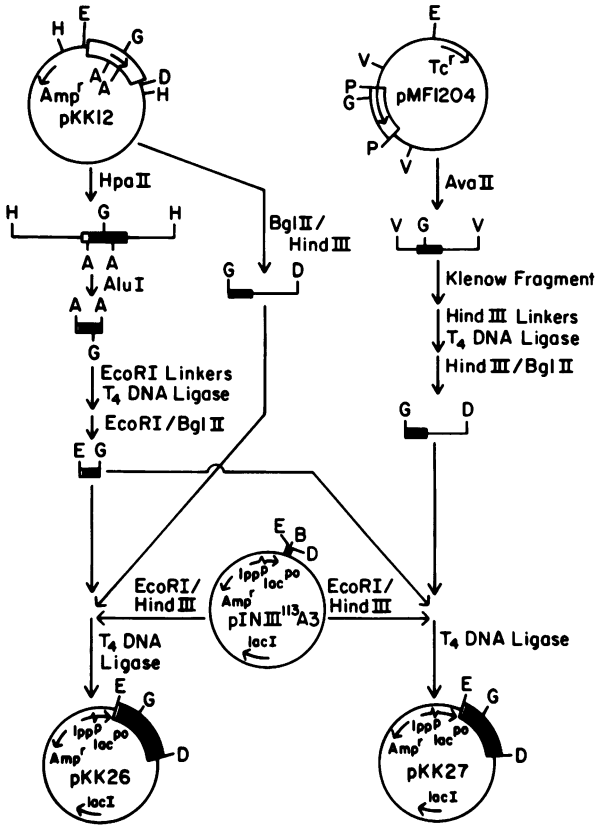

Figure 1 Strategy for construction of E.coli expression plasmids for a fusion MuIFN-α_4 (pKK26) and hybrid fusion MuIFN-α_4/α_2 (pKK27) genes. The coding regions were inserted into the expression plasmids behind the lpp promoter, under control of the lac^{po} region in pINIII^{113}. The coding regions of MuIFN-α_4 (pKK12) and MuIFN-α_2 (pMF1204) are indicated as arrows within the mouse DNA inserts (indicated by open boxes.) The fusion proteins made by these plasmids contain 15 amino acids more than the mature protein. The sequence of these extra amino acids is MetLysGlyGlyIleProTyrTyrTrpSerAla CysSerLeuGly. The first six are from the lpp gene and EcoRI linkers which were added to the coding regions; the remaining nine are signal sequence residues of MuIFN-α_4. A, AluI; D, HindIII; E, EcoRI; G, BglII; H, HpaII; P, PstI and V, AvaII. For the amino acid sequences of MuIFN-α_4 and -α_2, see references 1 and 8, respectively.

Expression of MuIFN-α genes in virus infected mouse cells

The relative levels of α mRNAs and kinetics of expression of the MuIFN-α genes in NDV induced mouse L-cells were

Table 1. Anti-viral activities of fMuIFN-α_4 and fMuIFN-α_4/α_2

Interferon	Source	Titer on[a] L-cells	Neutralization Titer[b]
------	pINIII^{113}	<2	------
fMuIFN-α_4	pKK26	16	1:1200
fMuIFN-α_4/α_2	pKK27	1600	1:600
MuIFN-α and -β mixture	NDV induced L-cells	--	1:2400

[a]Lysates of E. coli containing the bacterial expression plasmids were assayed as described in Materials and Methods. The titers are expressed in units/ml of bacterial culture.

[b]The neutralization titers are expressed as the highest dilution of goat anti-mouse IFN serum which neutarlizes 50 units of IFN. Neutralization were were performed as described in Materials and Methods.

examined by Northern and S1 nuclease analysis. Total RNA was isolated from L-cells at different times after infection with NDV and analyzed by Northern hybridization with the coding region of the MuIFN-α_2 cDNA. The results show that the MuIFN-α mRNAs reached peak levels in the induced cells by 10 hr after infection and no detectable levels were found 20 hr after infection (Fig. 2). Also, no detectable levels of MuIFN-α mRNAs were found in uninduced cells.

Since the MuIFN-α_2 probe used for the Northern analysis cross-hybridizes to all species of MuIFN-α mRNAs we have previously identified, the relative levels of each of the four cloned genes were determined by S1 nuclease analysis. Total RNAs isolated from infected cells at different times after induction were annealed with appropriate 5' end labeled DNA probes for each MuIFN-α gene (Fig. 2), digested with S1 nuclease, and the protected fragments were analyzed on polyacrylamide gels. RNA transcripts for three (α_4, α_5 and α_6) of the four MuIFN-α genes identified in the cluster, and for the α_2 gene were found in the infected cells (Fig. 3-4). An additional transcript of a new α gene (MuIFN-α_7) was detected with the 900 bp PvuII/EcoRI fragment of MuIFN-α_6; this new RNA gives a 98 bp protected fragment

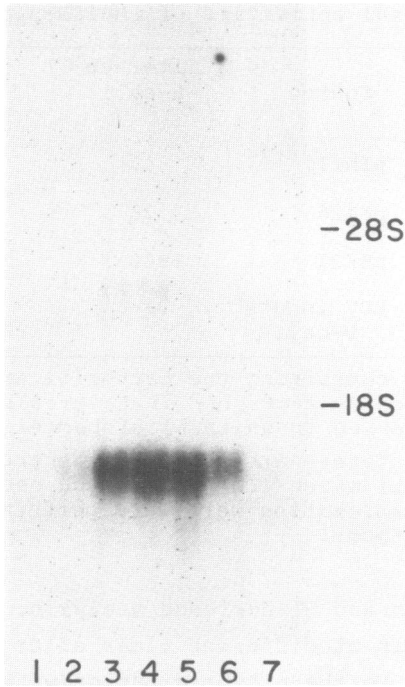

Figure 2 Northern analysis of Newcastle disease virus-induced L-cell cytoplasmic RNA with the MuIFN-α_2 BglII/HincII probe (8). RNAs were extracted at various times post-induction and analyzed for the presence of MuIFN-α mRNAs as described in Materials and Methods. Locations of 28S and 18S ribosomal RNAs are indicated on the right. Lanes 1-7; 0, 4, 6, 8, 10, 16 and 20 hrs post-induction.

with the α_6 probe, while the RNA transcripts of the MuIFN-α_6 gene give a 414 bp long protected fragment with the same probe. The S1 nuclease analysis further indicated that the MuIFN-α_4, -α_5 and -α_6 mRNAs have the cap site at the same position as the HuIFN-α_1 mRNA (14).

An additional S1 nuclease analysis was performed to determine whether the MuIFN-α_4 gene expressed in L-cells, (which originated from C3H mice) contains the same deletion as found in the α_4 gene in BALB/c mouse embryo DNA. A 5' labeled probe at the BclI site of MuIFN-α_4 was used in an S1 nuclease protection analysis of RNA isolated from NDV-induced L-cells 8 hr after infection. The BclI site is located in the coding region of α_4, downstream of the 15 bp deletion, and 544 bp

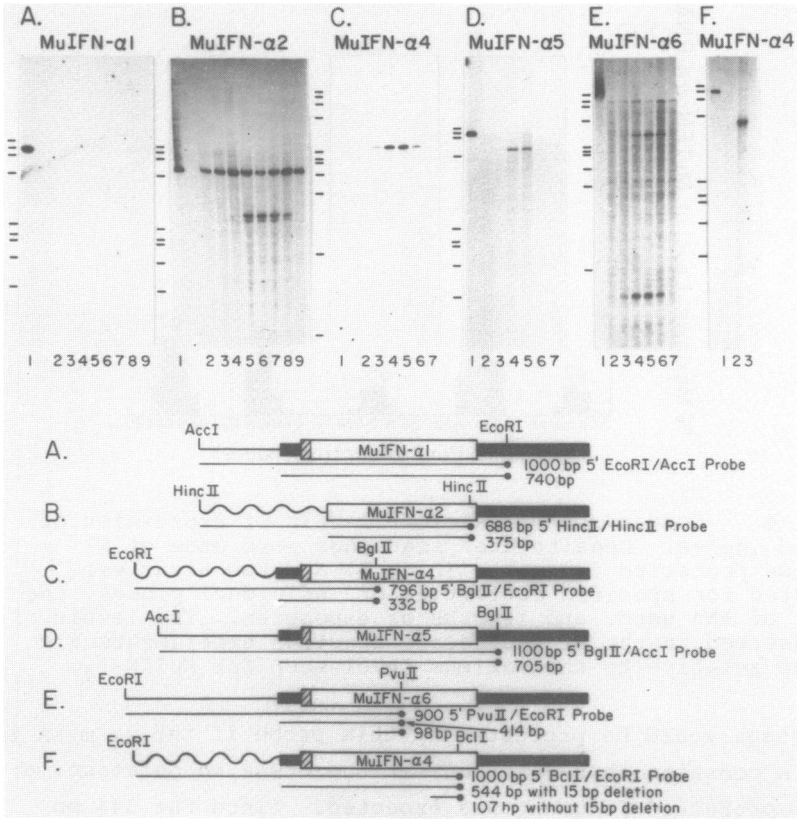

Figure 3 S1 nuclease protection experiments to detect MuIFN-α mRNAs in NDV-infected L-cells. 5' end-labeled probes specific for individual MuIFN- genes were prepared as described in Materials and Methods. After hybridization with total cytoplasmic RNA at 50°C and treatment with S1 nuclease, protected fragments were electrophoresed through 5% acrylamide, 7M urea gels. The autoradiograms in A-F represent different MuIFN-α genes and corresponding representations of the probes and expected protected fragments are indicated beneath the autoradiograms; 5' labeled-ends are indicated by filled circles (●). Lane 1 in A-F is the full-length DNA probe for each MuIFN-α gene. Lanes 2-9 in A and B are 10 μg of cytoplasmic RNA from 0, 4, 6, 8, 10, 12, 16 and 20 hr post-infection. Lanes 2-7 in C-E are 5 μg of cytoplasmic RNA from 0, 6, 8, 10, 16 and 20 hr post-infection. Lanes 2 and 3 in F are 5 μg of cytoplasmic RNA from 0 and 8 hr post-infection. Exposure times were 10d (A), 7d (B), 20 hr (C), 7d (D), 7d (E) and 65 hr (F). The positions of ^{32}P-labeled ϕX174 HaeIII fragments are indicated to the left of each autoradiogram and are 1373, 1078, 878, 603, 310, 281, 271, 234, 194, 118 and 72 bp in length.

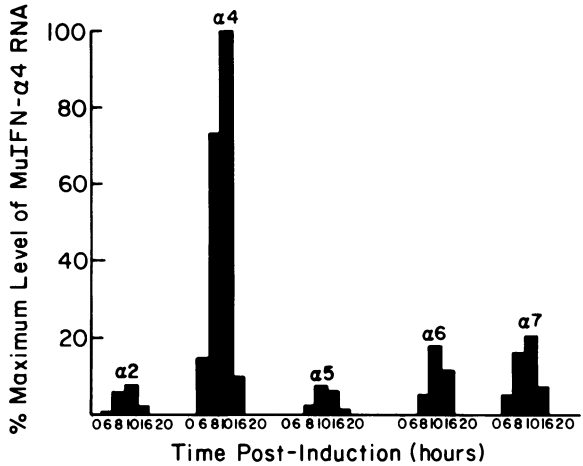

Figure 4 Comparison of relative levels of expression of MuIFN-α genes. Densitometer scannings were made of S1 nuclease protected fragments (Fig. 3) and the areas were corrected for specific activities of individual probes, the amount of RNA used, and lengths of exposures. The levels of RNA observed in the S1 nuclease protection experiments are plotted relative to the maximum level seen for MuIFN-α_4.

of message would be protected by this probe if the α_4 mRNA in L-cells contains this deletion; if there was no deletion, a 107 bp protected fragment was expected. Since the 544 bp protected fragment was detected (Fig. 3F), this shows that the MuIFN-α_4 gene present in L-cells (and in C3H mice) is identical to the deleted α_4 gene identified in BALB/c mice.

No transcripts corresponding to the MuIFN-α_1 gene were detected by the S1 analysis, even after two week exposures (Fig. 3A). Attempts to identify the MuIFN-α_1 mRNA in induced cells by Northern analysis using a unique fragment from the 3' untranslated region (1) were also negative. Also no detectable levels of RNA transcripts from the previously identified repeated intergenic fragment (1) were found in either uninfected or NDV infected L-cells (data not shown).

After corrections for specific activities of the individual probes, exposure times and the amount of RNA analyzed, it was found that the MuIFN-α_4 gene was expressed at levels 5-fold higher than α_6 and α_7, and 10-15 fold higher

than α_2 and α_5 (Fig. 4). All of the expressed genes reached peak levels of mRNA at 10 hr post-induction.

DISCUSSION

Both human and mouse α interferon genes are members of a related multigene family. The study of functional relationships between these closely related genes as well as modulation of their responses by various inducers may contribute to our understanding of the control of gene expression. Four murine α interferon genes were recently found to be closely linked (1); three of these genes show a high degree of homology both in the 5' end and coding regions while one of these genes (α_4) shows a higher degree of variability in these regions.

Several interesting findings with the MuIFN-α_4 gene were made, not only with respect to its genomic organization, but also in relation to its protein structure and expression. This gene contains a 15 bp in-frame deletion which removes 5 amino acids from its mature protein at positions 103-107. It was, therefore, of interest to examine how this deletion affects the biological activity of this protein. A bacterial fusion protein of this deleted gene had 100-fold less antiviral activity than a fusion protein in which the deleted region was replaced by a segment of normal length from MuIFN-α_2 (Fig. 1, Table I). The antiviral activity of the α_4/α_2 hybrid is similar to those of MuIFN-α_1, -α_5 and -α_6 when these are expressed using similar constructs in the same prokaryotic vector (Kelley and Pitha, unpublished observations).

Three of the four identified MuIFN-α genes are expressed in NDV infected L-cells. However, large differences in the relative levels of the individual MuIFN-α mRNAs were detected. Surprisingly, 5 to 15-fold higher levels of α_4 mRNA, which contains a 15 nucleotide deletion in its coding region, were detected. This α_4 gene is the only gene in the cluster that does not have the repeated sequence present in the upstream region of the 5' end flanking sequence. Thus if this repeated sequence has any role in the regulation

of MuIFN-α gene expression, it would be a negative one. The DNA sequences of the promoter regions of these α-interferon genes were further examined to determine whether the higher levels of expression of MuIFN-α_4 could be correlated with differences in DNA sequences in this region. A comparison of the MuIFN-α promotors to a consensus sequence of the HuIFN-α promoters reveals that there are regions which are highly conserved between them (Fig. 5), including the GAAAG sequence found in viral and cellular enhancers (15). There are, however, 43 positions at which the MuIFN-α_4 sequence is dissimilar from the other mouse promoters (Fig. 5); 19 of these changes are at positions where the three remaining mouse sequences are the same as the human consensus sequence. In addition, the MuIFN-α_4 promoter contains a G rich region not seen in the other MuIFN-α promoters (Fig. 5). Thus the high expression of MuIFN-α_4 may be related to alterations in the promoter region. The possibility exists that mutations in the promoter region of this gene which allowed increased expression after induction were not under a high degree of evolutionary selection due to the lower specific activity of its encoded deleted protein. However, since we do not know whether the stability of IFN mRNAs is affected by the interferon proteins synthesized, we can not eliminate the possibility that the accumulation of the α_4 mRNA may be due to post-transcriptional events and related to the synthesis of an altered interferon peptide.

It was reported that MuIFN-α_1 mRNA could be detected in NDV-induced Ehrlich ascites tumor (EAT) RNA by S1 nuclease protection experiments, although at 4-16 fold lower levels than MuIFN-α_2 (16). Using the same MuIFN-α_1 probe as Shaw, et al. (16) for S1 nuclease analysis, we could not detect any mRNA for this gene in NDV-induced L-cell total cytoplasmic RNA, even though mRNA for MuIFN-α_2 could be seen (Fig. 3A-3B). No polyadenylation signal was found within 572 bp of 3' untranslated sequences for MuIFN-α_1 (1) and the possibility exists that even if this gene is expressed, it may not be polyadenylated properly, although this gene has been detected in poly(A^+) RNA from NDV-infected EAT cells (16). It is

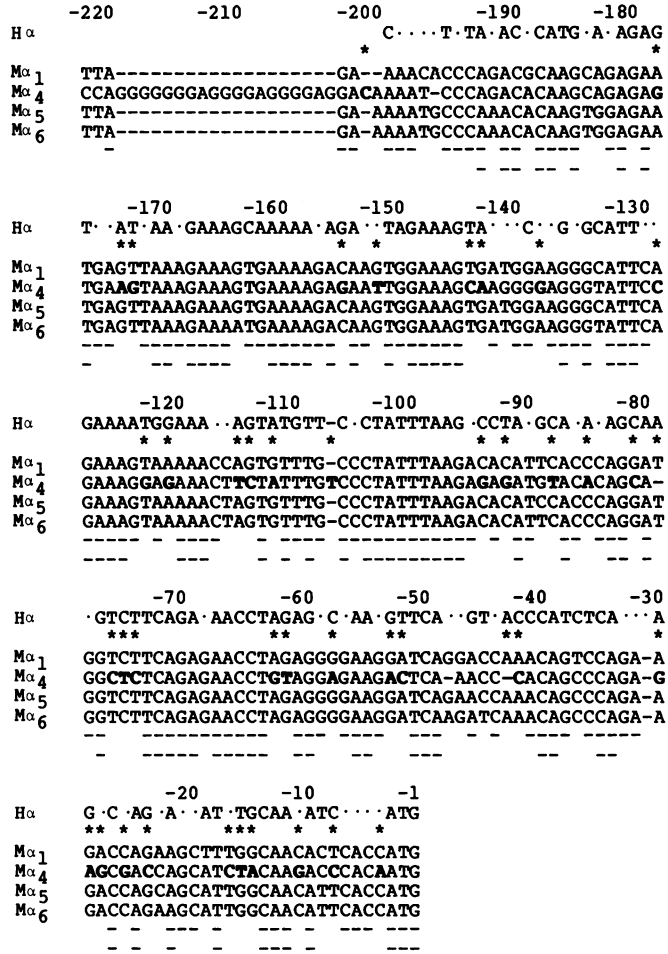

Figure 5 Comparison of MuIFN-α promoters to a HuIFN-α promoter consensus sequence. 1) The human consensus sequence was derived from 7 HuIFN-α 5' sequences (17); a common base was assigned to a position if that base is present in at least 6 of the 7 promoters. Dots (.) indicate positions at which there is no common nucleotide. Sequences for the 5' regions of MuIFN-α_1, MuIFN-α_4, MuIFN-α_5 and MuIFN-α_6 are aligned with the human consensus sequence for maximum homology. A single underline beneath the mouse promoters indicates positions where there is a common nucleotide; double underlines indicate positions where a common nucleotide in the mouse sequence is the same as the human consensus. Asterisks (*) above the mouse sequences indicate positions where the MuIFN-α_4 promoter is different from the other MuIFN-α promoters. The sequence GTGAAAAG at positions -164 to -157 is very similar to sequences which have been identified in viral and cellular enhancers (15).

possible that the amount of MuIFN-α_1 mRNA in infected L-cells was below the level of detection by our S1 nuclease analysis.

The data show an interesting difference between the inducibility of α-IFN genes in mouse and human cells. While in human cells the α-interferon genes do not seem to be effectively induced in infected fibroblasts or epithelial cells (18,19) and their expression seems to be limited to the cells of lymphoid origin (14,19,20), the induction of expression of MuIFN-α genes does not show this cell type specificity (18,16). The relative levels of different types of HuIFN-α mRNA in infected cells also reveal a large variability; however, no obvious correlation between the levels of α mRNA expression and 5' flanking sequences or protein activities of the α-interferon peptides emerges. It will be interesting to examine in reconstruction experiments whether the polypurine rich oligomer present in the 5' end region of the MuIFN-α_4 gene can increase expression of the α-interferons driven by poorly inducible promoters, and whether the α_4 gene is preferentially expressed in other types of mouse cells as well.

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