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Specific antiviral activities of the human α interferons are determined at the level of receptor (IFNAR) structure

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Differences in activity among the family of human IFNs α are much reduced if these ligands are assayed on bovine cells. In particular, the activity of IFN αD is much higher on bovine than on human cells. To examine these differences, the bovine counterpart of the human IFNAR has been cloned and expressed in a human cell line. The transfected cell line now recognizes the human IFN αD as a high-specific-activity IFN subtype, indicating that the differences in sensitivity between the bovine and human cells to the human IFN α lie in the structure of the IFNAR chain rather than in the other components of the functional receptor.

Interferon; Interferon receptor; Bovine; Transfection; Cytokine receptor family

1. INTRODUCTION

Apart from their antiviral activities, by which they were first described, the interferons (IFNs) show negative effects on the proliferation of cultured cells and possess basic immuno-modulatory effects at the level of B-cell, T-cell and macrophage activation [1]. By itself the human type I IFN family consists of 15–20 species of IFN α and IFN β encoded by as many structural genes. The reasons for the evolutionary selection of such a gene family encoding type I IFNs are largely unknown but there seems to have been pressure to retain more than one type I IFN gene in mammals [2,3]. An examination of the familial differences is therefore important in assessing the functional significance of this multiplicity of related ligands.

On human cells, all the IFN α and β species compete fr the same receptor system on the cell surface [4,5] but the individual subtypes show different specific antiviral or antiproliferative activities [6–8]. It has been shown that the quantitative differences between the IFN subtypes are related to their binding behaviors at the cell surface [9]. In particular, IFN αD shows a much lower binding affinity for human membrane receptors than either IFN αA or αB [10]. However, on bovine cells, human α IFNs exhibit a pronounced cross-reaction and all subtypes, including αD, have the same high specific activities and behave similarly at the receptor level [6–8,11–13]. It is thus reasonable to assume that, compared to the human receptor, the bovine functional receptor unit exhibits critical differences which allow it to bind all human α IFN subtypes with the same high affinity.

One component of both the human and the mouse type I IFN functional receptor unit has been already characterized [14,15]. This transmembrane protein, called IFNAR for IFN α receptor, is structurally related to other members of the cytokine receptor superfamily characterized by a 200 amino acids domain (D200) subdivided in two 100 amino acids subdomains (SD100A and B) [16–18]. The predicted folding of each SD100 is the 7 β strands (S1–S7) of the immunoglobulin constant domain [16,17]. When expressed in heterospecific cells, IFNAR participates in the functional receptor unit for a limited number of type I IFNs but is likely implicated in the mediation of the binding and activities of all type I IFNs when expressed in a homospecific background [15,19]. The receptor system that is emerging is thus not simple, the results strongly suggest that an additional membrane component must come into a proper steric contact with IFNAR for a high-affinity site to be established.

In order to examine the role of the IFNAR chain in the peculiar behavior of the bovine receptor for human IFN α subspecies, we have cloned and sequenced the bovine counterpart of the human IFNAR from MDBK cells. We show that, when expressed in human cells, the bovine receptor is capable of high-affinity binding and high specific antiviral activity for all human IFN α subspecies tested, including the human IFN αD.

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Abbreviations: IFN, interferon; IFNAR, type I interferon receptor.

2. MATERIALS AND METHODS

2.1. *Nucleic acids cloning and analysis methods*

Standard procedures were as described [20]. An oligo(dT)-cDNA library was constructed in the λ ZAP II phage vector (Stratagene) from poly A⁺ RNA of MDBK cells using the cDNA synthesis system from Amersham. Low-stringency hybridization conditions were according to Howley et al. [21] as previously described [15]. High-stringency hybridization conditions with the probe indicated in the text were according to Church and Gilbert [22]. All DNA fragments were cloned into Bluescript KS II⁺ from Stratagene or into the mammalian expression vector pVADN1 [15]. DNA sequencing and handling were as described [18].

2.2. *IFNs*

Human IFNs αB (α8) and αD (α1) were a gift from Ciba-Geigy Ltd. Human IFN αA (α2c) was from Bender+Co GmbH, Wien. The preparation of IFNs labeled with ^{125}I was as described [23]. Antiviral titers were estimated by a cytopathic inhibition assay using vesicular stomatitis virus or encephalomyocarditis virus as challenge virus [24].

2.3. *Cell lines and transfections*

Madin–Darby bovine kidney (MDBK, ATCC 6071) and human epithelial HEp 2 cell lines (ATCC CCL 23) were grown in Dulbecco modified Eagle's medium supplemented with 10% fetal calf serum. The HEp 2 cell line was transfected by the calcium phosphate precipitation technique [25]. Stable transfected clones were selected in the presence of G418 (Gibco) at 1 mg/ml.

2.4. *Binding experiments*

Experimental procedures for binding experiments with iodinated IFNs were described in a previous article [14]. Briefly, they were carried out at 37°C on $1 \cdot 10^6$ cells/well plated on six-well plates. Background counts of non-specific binding were estimated by incubating controls with a 100-fold molar excess of the appropriate unlabeled IFN.

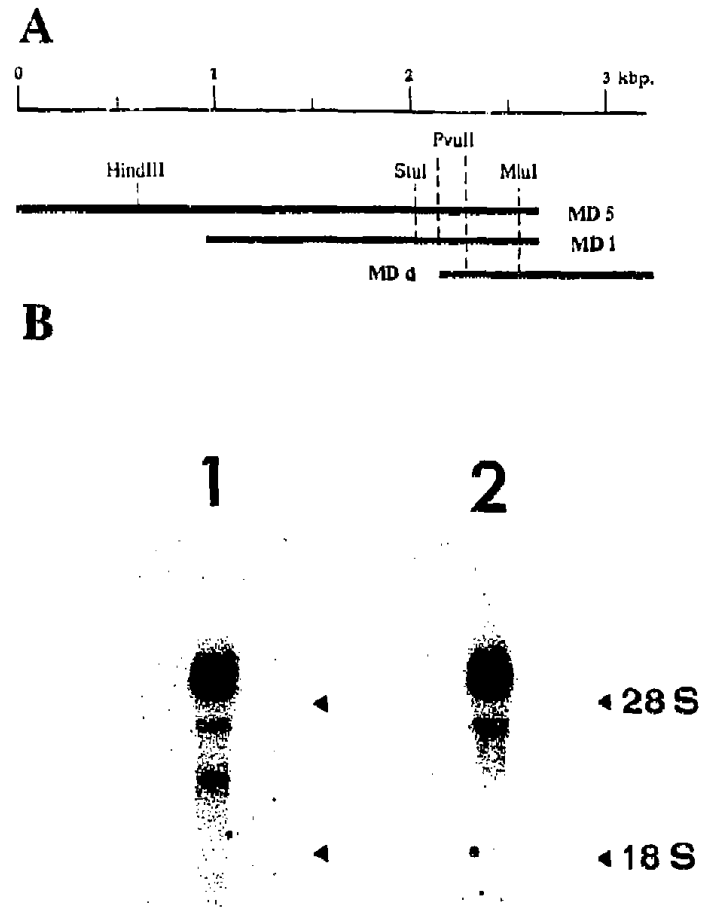

Fig. 1. A: schematic diagram representing the various overlapping cDNAs; B: Northern blot analysis of 10 μg of RNA from bovine MDBK cells hybridized with MD5 (lane 1) or the 3′ sequence of MDd (lane 2).

3. RESULTS AND DISCUSSION

3.1. *Cloning of IFNAR cDNA from MDBK cells*

Oligo (dT) primed cDNAs synthetised from poly(A)⁺ RNA of MDBK cells were cloned in the lambda Zap II phage vector. $5.6 \cdot 10^6$ independent clones of this library were screened with two probes derived from the human IFNAR cDNA [14]. One probe encodes the first 200 amino acids of the mature extracellular part, the other encodes the 100 amino acids that constitute the intracytoplasmic domain of the human receptor. Low stringency hybridization conditions used according to Howley et al. [21] resulted in the identification of two phage clones: MD1 andMD5. Restriction map and sequence analysis revealed that they contained overlapping cDNA inserts recognized by the human probes. These cDNAs are schematically depicted in Fig. 1A. Their sequences have been deposited in the EMBL data library (accession No. X68443) and are available upon request. The MD5 sequence consists of 2,645 bp plus a 28-residue stretch of adenine 21 bp downstream of a polyadenylation signal AATTAAA, and contains an open reading frame of 1,680 bp starting at nucleotide 125 and closed at both 5′ and 3′ ends by termination codons. When hybridized on RNA from MDBK cells on Northern blot, the 5′ *Hin*d III fragment of the cDNA MD5 (nucleotide 1 to 607) detects three transcripts of 6 kb, 4 kb and 2.7 kb (Fig. 1B, lane 1). The same result was obtained using a probe corresponding to the 3′ end of the MD5 cDNA (data not shown). We thus considered the possibility that the length discrepancy between the cDNA MD5 (2,7 kbp) and the mRNAs of 6 and 4 kb was due to incomplete 5′ or 3′ regions. The existence of an additional sequence in the 5′ region of MD5 cDNA was tested by the polymerase chain reaction with single-sided specificity as described [15], but we were unable to show the presence of such a sequence, even if RNA from MDBK cells was denatured with methyl mercury prior to the reverse transcription. However, a search for 3′ sequences of MD5 cDNA was successful: the MDBK cDNA library was rescreened with a *Stu*I–*Mlu*I restriction fragment of the MD5 cDNA (nucleotide 2025 to 2560). A new clone (MDd) with a 1,1 kbp insert overlapping with MD5 was isolated; it is schematically depicted in Fig. 1A. Its 5′ extremity corresponds to nucleotide 2171 on the sequence of MD5 and is identical to MD5 up to nucleotide 2645, just upstream of the poly A stretch of MD5. At this position, MDd contains an additional non coding 585 bp sequence (EMBL ac-

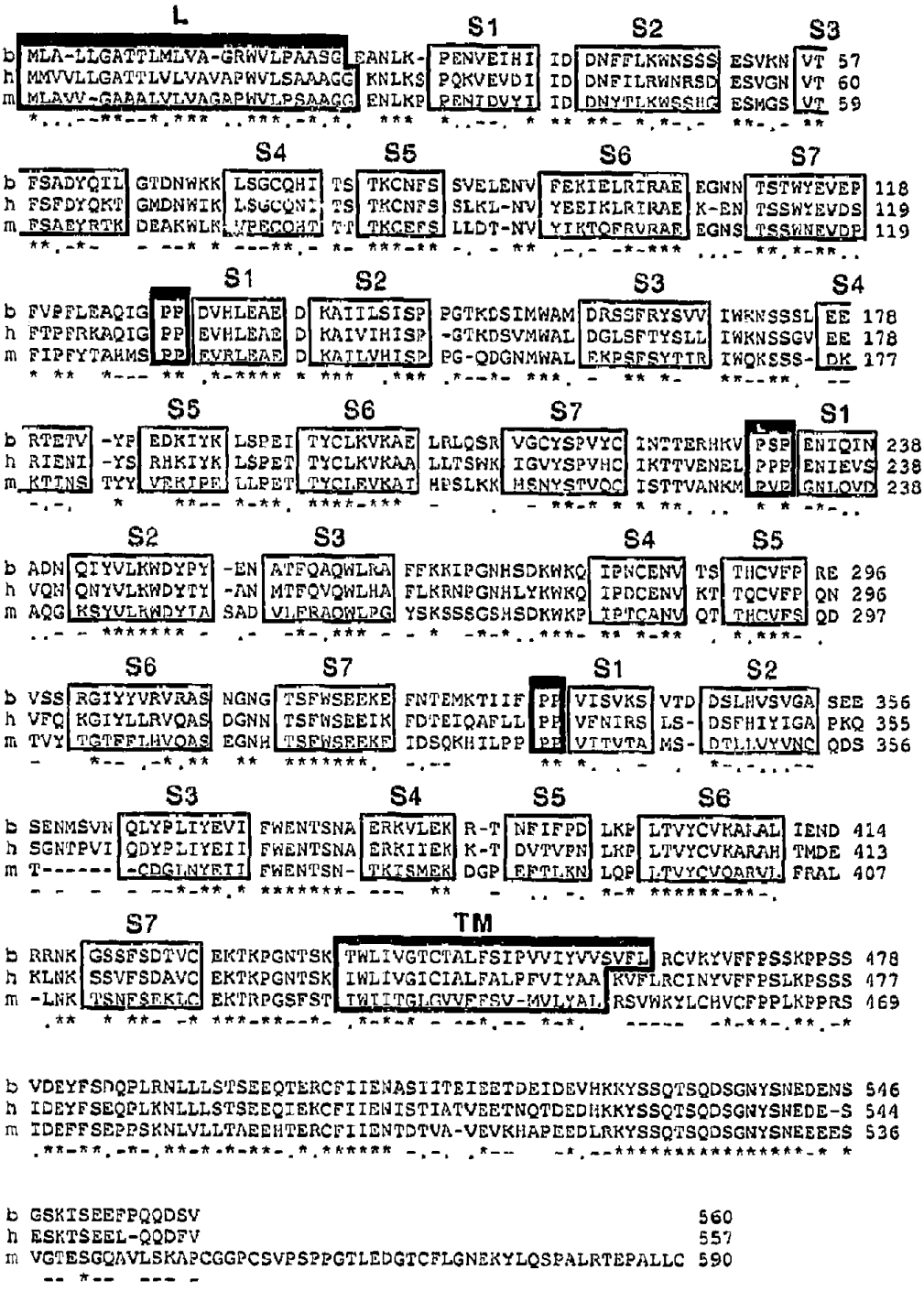

Fig. 2. Predictive alignment of protein sequences of the bovine (b), human (h) and murine (m) IFNAR. The amino acids are represented according to the one letter code. Leader peptides (L) and transmembrane regions (TM) are stressed by large heavy boxes. Small heavy boxes with the dipeptide 'PP' correspond to the hinge regions between SD100A and SD100B and between SD100A' and SD100B'. The tripeptide small heavy box corresponds to the hinge region between the two D200. Predicted β strands are boxed and numbered S1–S7. SD100A corresponds approximately to positions 30–130, SD100B corresponds approximately to positions 130–230, SD100A' corresponds approximately to positions 230–335, SD100B' corresponds approximately to positions 335–430. Stars indicate positions that are conserved in the three sequences. Lines indicate positions that are conserved only between human and bovine sequences. Dots indicate the positions that are conserved between the murine and only one of the other two sequences.

cession no. X68443). In order to demonstrate that the length discrepancy between the three transcripts detected with MD5 is due to the presence of sequences in the 3' region to the shorter 2.7 kb transcript, we hybridized the 3' sequence of MDd (nucleotide 2792 to 3194) on RNA from MDBK cells (Fig. 1B, lane 2). Indeed, this probe detects only the two longer transcripts of 6 and 4 kb but not the 2.7 kb mRNA. The three tran-

scripts detected with MD5 can thus be explained by alternative use of polyadenylation sites which generate different lengths of the 3′ untranslated regions.

This result strongly suggests that the cDNA MD5 corresponds to the 2.7 kb mRNA and that it contains the complete open reading frame encoding the bovine IFNAR. Despite the lowest abundance of the 2.7 kb transcript, the first screening of the cDNA library with human probes did not allow us to isolate the two longer cDNAs. Because of the efficiency of reverse transcription and the location of the probes, it is likely that cDNAs originating from the shortest transcript were selectively cloned.

3.2. *Structural comparison of the bovine, human and murine IFNARs*

The open reading frame of the cDNA MD5 encodes a 560 amino acid protein showing the typical structure of an interferon receptor, including a 24 amino terminal hydrophobic stretch canonical for a signal peptide [26]. Another stretch of 25 hydrophobic residues bisect the polypeptide into a putative extracellular part of 413 amino acids and a putative intracytoplasmic region of 98 amino acids. 15 characteristic motifs of *N*-linked glycosylation are found, including 2 in the intracellular domain.

Fig. 2 presents an alignment between the bovine and the two other known IFNAR sequences [14,15]. This alignment has been obtained by the clustal program [27] and refined by hydrophobic cluster analysis [28] to ensure the conservation in the bovine and mouse IFNARs of the β strands predicted by Bazan [16] and Thoreau et al. [17] for the human IFNAR and confirmed recently by the determination of the structure of another member of the growth hormone/prolactin/cytokine receptor family [29]. Gaps have been found in the predicted loops rather than in the predicted β strands. It is apparent from this alignment, that the predicted folding of the human IFNAR is conserved for the bovine and murine receptors with four immunoglobulin-like constant-domain structural units (SD100A, B, A′ and B′). All cysteine positions are conserved between the human and the bovine receptor except Cys^{214} which appears to be isolated in the S7 β strand of SD100B of the bovine IFNAR. Three potential *N*-linked glycosylation sites are conserved in the three receptors. It is interesting to note that both S6–S7 loops of the two SD100A contain a conserved glycosylation sequence and that the third conserved putative site is in the predicted S3–S4 loop of SD100B′. The intracytoplasmic domain of the bovine IFNAR has the same length as the human, rather than the longer intracytoplasmic domain exhibited by the mouse IFNAR. The most obvious point in the alignment of this domain is the stretch of 16 amino acids perfectly conserved in the three species which could be implicated in interaction with intracytoplasmic components of the functional receptor unit.

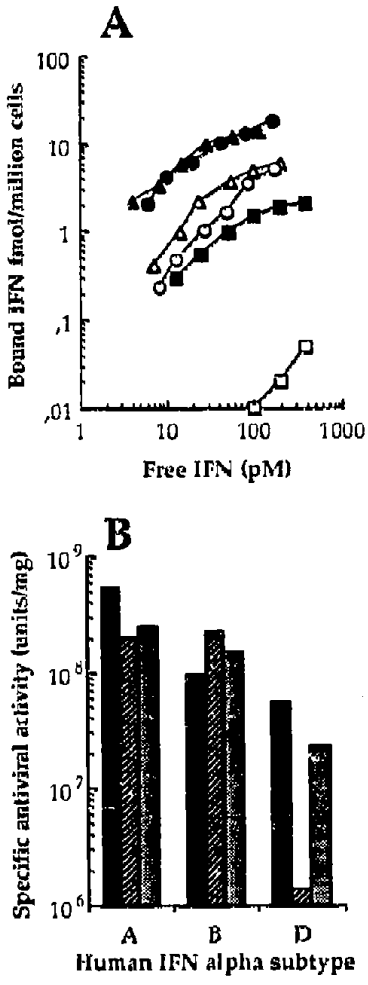

Fig. 3. A: binding activity of parental human HEp 2 cells (open symbols) or human HEp 2 cells transfected with the bovine IFNAR (closed symbols) for the human IFN αA (circles), αB (triangles) and αD (squares); B: specific antiviral activity of human IFNs αA, αB and αD assayed on bovine MDBK cells (black column), parental human HEp 2 cells (striped column) or human HEp2 cells transfected with the bovine IFNAR (dotted column).

3.3. *Expression of the bovine IFNAR in human cells*

The open reading frame containing the 5′ *Pvu* II fragment of MD5 cDNA (nucleotides 1 to 2150) was cloned downstream of the adenovirus 2 major late promoter in the mammalian expression vector pVADN1 to obtain pVADN1MD56. This plasmid was transfected into the human HEp 2 cell line and stable transfectants were selected in the presence of G418. One transfectant named HBB64 was selected on the basis of the expression of the bovine IFNAR mRNA and was further analysed.

Fig. 3A shows the IFN binding of various human α IFN subtypes on proliferating parental HEp 2 cells (open symbols) and on the transfected HBB64 clone (closed symbols) after 1 h of contact between IFNs and cells, a time corresponding to a maximum uptake for the IFN concentration range studied. The binding was

measured at 37°C because it has been shown that under these conditions, cellular activities due to IFNs map in a one-to-one fashion to the activities of the IFN-receptor complex [9,24,30]. The curves indicate that the bovine IFNAR provides high-affinity binding sites for the human IFN αD on the HBB64 clone whereas parental HEp2 cells bind this IFN with lower affinity as already described for the human Daudi cell line [9]. The level of IFN uptake attained by IFNs αA and αB on the human cell line is now attained by IFN αD on human cells expressing bovine IFNAR. Correlating with the uptake, the specific antiviral activity of IFN αD on human cells expressing the bovine IFNAR, approaches the activity measured on bovine MDBK cells in contrast to the specific activity of αD on human parental cells which is 100-fold lower. It is also remarkable that the uptake of IFNs αA and αB is 0.5–1 log higher on cells expressing both human and bovine receptors than on the parental human cells. As shown in Fig. 3B, this higher binding activity of the HBB64 clone does not confer a higher biological activity for IFN αA and αB suggesting that the transducing machinery can be saturated.

These results show that the bovine counterpart of the human IFNAR participates, when expressed into human cells, in a functional receptor unit capable of recognizing the human IFN αD as a high specific activity IFN α subtype. It is thus likely that the molecular determinants of the differences in the specific activities of the human interferon α subtypes on human cells lie in the sequence of the IFNAR chain.

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