Promoter structure of the *Mx*A gene that confers resistance to influenza virus

Kohji Nakade[1], Hiroshi Handa, Kyosuke Nagata*

Department of Biomolecular Engineering, Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama 226, Japan

Received 17 September 1997; revised version received 7 October 1997

Abstract The human MxA protein is one of the interferon-inducible proteins that inhibits multiplication of influenza virus and other viruses. To clarify the control mechanism of its expression, we prepared a series of mutant *Mx*A promoters and identified a 30 nucleotides long *cis*-acting interferon-responsive element by transient transfection assay. Its nucleotide sequence is somewhat similar to that of ISRE (interferon-stimulated response element), suggesting that the regulation of MxA mRNA synthesis is under the control of some ISRE binding factor such as ISGF-3 (interferon-stimulated gene factor-3).

Key words: MxA; Interferon; Transcription promoter; Influenza virus; Reverse transcription-polymerase chain reaction; Luciferase assay

1. Introduction

Interferon (IFN) induces a variety of proteins to establish an anti-viral state. The human MxA protein is one of these IFN-inducible proteins that confers resistance to influenza virus. Studies on the *Mx* gene started with the finding of a mouse strain resistant to influenza virus infection [1]. It has been shown that the gene encoding the murine 72-kDa Mx1 is located on chromosome 16 [2–4]. More recently, the human 76-kDa MxA protein was identified as a structural and functional homologue of the murine Mx1 protein [5,6]. The Mx proteins are present in most vertebrates but the anti-virus spectra are different. For example, the murine Mx1 protein inhibits proliferation of influenza virus but not vesicular stomatitis virus (VSV), while the human MxA protein is effective for both viruses. The human MxB protein is structurally similar to the MxA protein, although the anti-influenza virus property is much lower than that of MxA [7–12]. A common trait is that Mx proteins are synthesized in response to stimulation by IFN-α or -β but not by IFN-γ in a limited number of organs, i.e. liver cells, peripheral blood cells and nerve cells. The Mx protein may play a role to evade the lethal destruction of tissue with its acute induction after virus infection.

The mechanism of IFN-dependent induction of Mx proteins remains unclear, although the three motifs similar to that of ISRE (IFN-stimulated response element) have been found in its transcription regulatory regions [13,14]. In this paper, we further investigated the region required for transcription of MxA mRNA and identified a minimum essential element necessary for its induction by IFN.

*Corresponding author. Fax: (81) (45) 924-5940.
E-mail: knagata@bio.titech.ac.jp

[1]Present address: 2nd Pharmaceuticals Laboratory, Yokohama Research Center, Mitsubishi Chemical Corporation, Yokohama, Japan.

2. Materials and methods

2.1. Detection of MxA mRNA by RT-PCR

Total RNA was prepared from 5×10^6 cells that had been maintained for 16 h in growth medium containing 1 kU/ml of IFN-α (Hayashibara Biochemical Laboratory Inc.). For detection of *Mx*A mRNA, reverse transcription was performed according to the manufacturer's instructions (Superscript Gibco) using primer 1 (Fig. 1A) complementary to MxA cDNA [5,6]. Polymerase chain reaction (PCR) was performed with primers 2 and 1. Amplification involved denaturation at 94°C for 1 min, primer annealing at 55°C for 2 min, and extension at 72°C for 3 min.

2.2. Cloning of MxA promoter

Genomic DNA of human glioblastoma T98G cells digested with *Hin*dIII was separated by agarose gel electrophoresis and DNA around 1.9 kbp long fragments was isolated. A genomic DNA mini-library was constructed by cloning the 1.9 kbp long DNA fragments into the *Hin*dIII site of Bluescript SK(+). To prepare the DNA probe, PCR amplification was performed using 1 μg of genomic DNA of T98G cells and two synthetic oligonucleotides, 5′-CCGTTTCCACC-CTGGAGAGGCCAGATGAGC-3′ and 5′-GTTAGCATCACTG-GACTCTACTTCCAGTA-3′ corresponding to nucleotide positions 53–82 and 1459–1488 of the *Mx*A genome DNA, respectively [14]. After screening of about 10 000 colonies [15], two positive clones that contained 1.9 kbp long DNA fragments including the 0.6 kbp upstream region from the *Mx*A transcriptional start site were obtained.

2.3. Construction of MxA promoter-luciferase reporter plasmids

The cloned 1.9 kbp long DNA fragment was digested with *Fsp*I and *Hin*dIII. The 0.6-kbp fragment was subcloned into the *Sma*I site of PGV-B (TOYO Ink) (Fig. 2A, pMxluc1–643). Then pMxluc1–643 was digested with *Apa*I, and briefly treated with *Bal*31. The vector fragments containing mutated *Mx*A promoter sequences were cut off at the *Sca*I site in the region that confers ampicillin resistance. The *Sca*I–*Sma*I large fragment was isolated from the original luciferase expression vector PGV-B. Finally, mutated *Mx*A promoter-luciferase DNA fragments and the *Sca*I–*Sma*I large fragment were ligated and selected by ampicillin resistance. DNA sequences were confirmed by the dideoxy-mediated chain-termination method [15].

2.4. Transfection and luciferase assay

T98G cells logarithmically grown in Dulbecco's modiefied Eagle medium (DMEM) supplemented with 10% FCS were trypsinized and resuspended in DMEM at a concentration of 3×10^6 cells/0.8 ml. Then 13 μg of *Mx*A promoter-luciferase plasmid was added to the cell suspension and immediately electroporated at 1160 mF and 260 V in high ohm (Bio-Rad Gene Pulser). The transfected cells were maintained for 36 h. The induction was carried out by adding IFN-α at a concentration of 1 kU/ml and the luciferase activity was measured according to the manufacturer's instructions (TOYO Ink) after several hours stimulation.

3. Results

3.1. Expression of MxA mRNA in T98G cells

To confirm the IFN-inducible synthesis of *Mx*A and *Mx*B

PII S0014-5793(97)01372-0

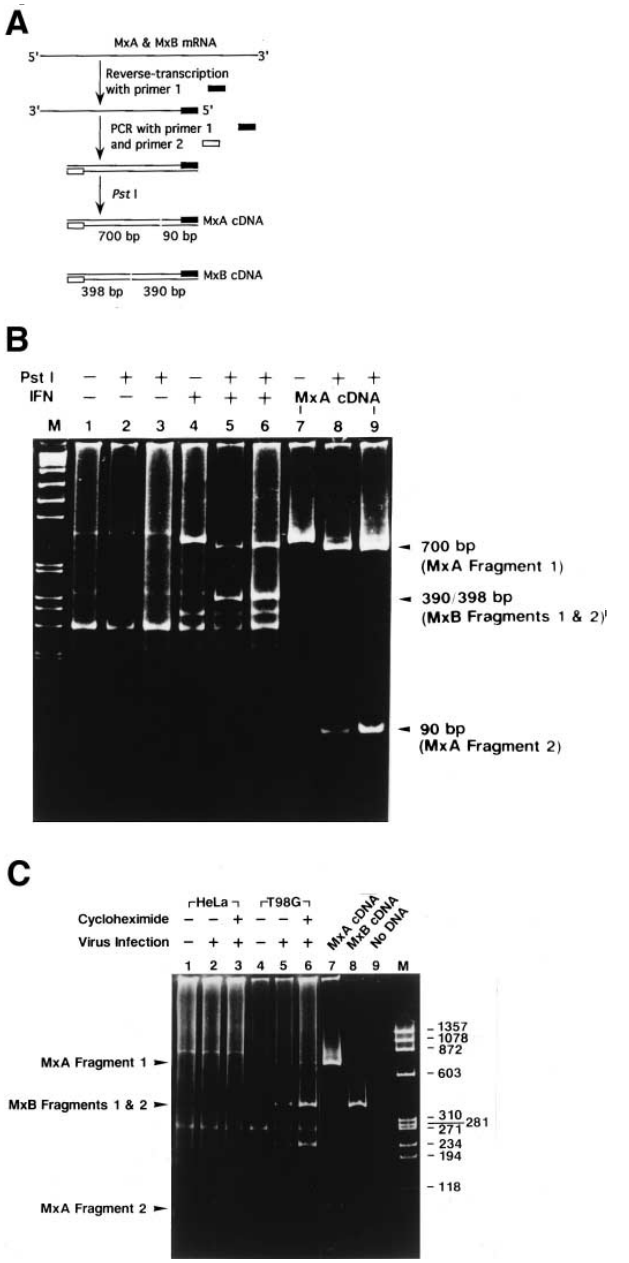

Fig. 1. Expression of human *Mx*A mRNA. A: The strategy for detection of *Mx*A mRNA. Closed and open boxes indicate oligonucleotides for primer 1 (5′-TCCAGGAGAACTCTGAAATATGGATG-3′) and primer 2 (5′-GCCCCTGCATTGACCTCATCGACTC-3′), respectively [5,6]. B: IFN-inducible expression of human *Mx*A mRNA. Total RNA extracted from either IFN-α-treated (lanes 4, 5, and 6; 1×10^4, 0.5×10^4, and 1.5×10^4 cell equivalents, respectively) or nontreated (lanes 1, 2, and 3; 1×10^4, 0.5×10^4, and 1.5×10^4 cell equivalents, respectively) T98G cells and pET3a-MxA plasmid DNA (lanes 7, 8, and 9; 5, 2.5, and 7.5 ng) were used as template. PCR products were digested with *Pst*I (lanes 2, 3, 5, 6, 8, and 9) and subjected to electrophoresis in 2% agarose gel and visualized by ethidium bromide staining. Lane M indicates DNA size markers. C: Induction of human MxA mRNAs by influenza virus infection. HeLa cells and T98G cells were infected with influenza virus at m.o.i. of 10 (lanes 2, 3, 5, and 6) and incubated for 12 h in the presence (lanes 3 and 6) or the absence (lanes 1, 2, 4, and 5) of 100 μg/ml cycloheximide. Total RNAs (0.5×10^4 cell equivalents) and 5 ng of pET3a-MxA and phuMxB17 plasmids were used as templates for RT-PCR. The PCR products were subjected to electrophoresis in 2% agarose gel and visualized by ethidium bromide staining. Lane M indicates DNA fragments of ϕX174 DNA digested with *Hae*III as size markers.

mRNAs in the human glioblastoma T98G cells, semi-quantitative determination of the mRNA level was carried out by the RT-PCR method (Fig. 1). Synthetic primers 1 and 2 correspond to the conserved regions between *Mx*A and *Mx*B genes (Fig. 1A,B) so that both mRNAs can be amplified and similar sizes of PCR products, 790 bp long DNA for *Mx*A mRNA and 788 bp long DNA for *Mx*B mRNA, can be synthesized [5,6]. Digestion of PCR products with *Pst*I was carried out to discriminate both species (Fig. 1A). Fig. 1B shows the results of this protocol for total RNA extracted from either IFN-α-treated (lanes 4–6) or untreated (lanes 1–3) T98G cells. Synthesis of both *Mx*A and *Mx*B mRNAs was induced by treatment with IFN. Under the same condition no detectable *Mx* mRNAs were observed in HeLa cells (data not shown). In this line, the level of influenza virus production in T98G cells was much less than that in HeLa cells (data not shown). In addition, the synthesis of *Mx*A and *Mx*B mRNAs is induced by influenza virus infection in the absence and even in the presence of cycloheximide (Fig. 1C), suggesting that there may exist not only an IFN-responsive element(s) but also a regulatory element responsible for virus infection for transcriptional regulation.

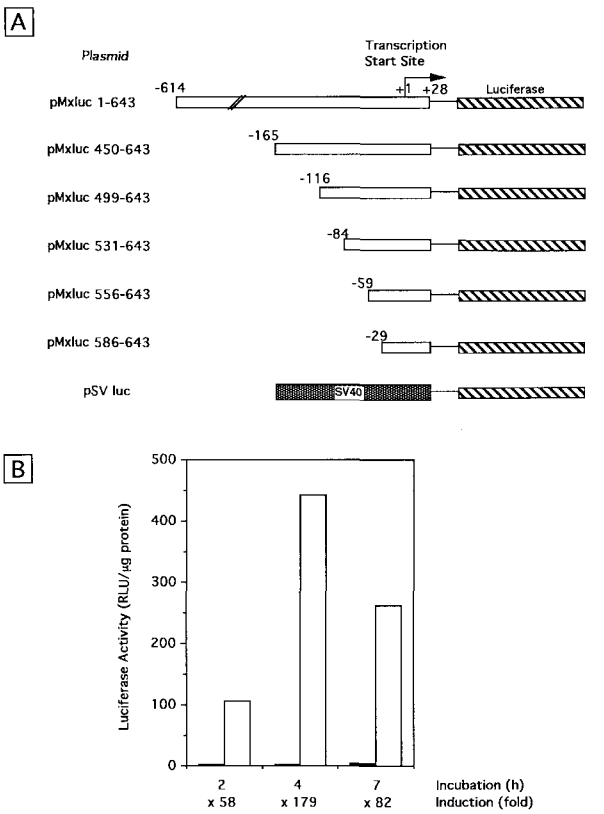

Fig. 2. IFN-inducible expression of the luciferase driven by the *Mx*A promoter. A: The schematic representation of the constructs of mutated *Mx*A promoters used for the luciferase transfection assay. Nucleotide numbers indicate the positions relative to the transcription start site. The plasmid pSVluc contains the SV40 enhancer-promoter. B: Time course of IFN-dependent transcription from the *Mx*A promoter. The pMxluc1–643 plasmid was transfected into T98G cells and incubated for 36 h. Either 1 kU/ml of IFN-α (open box) or mock solution (closed box) was added. The cells were lysed and their luciferase activities were measured after further incubation for 2, 4 and 7 h. 'Fold' gives the ratio of the luciferase activity in IFN-treated cells over that in untreated cells.

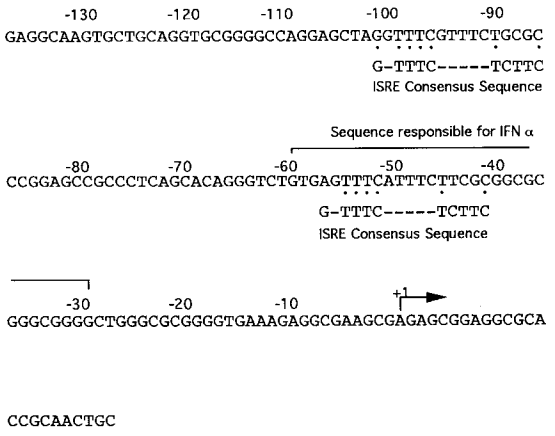

Fig. 3. The DNA sequence of the *Mx*A promoter region. Nucleotide numbers indicate the positions relative to the transcription start site shown by bar with arrowhead. Two ISRE-like sequences are found in about −50 and −90 nucleotides upstream from the transcription start site. The sequence responsible for IFN-α determined by this study is indicated by a bracket.

3.2. *Determination of cis-acting regulatory elements for transcription*

In order to map a region(s) responsible for transcription induction by IFN, we prepared a series of deletion mutants of the *Mx*A gene promoter (Fig. 2A). When plasmid pMxluc1–643 is introduced into T98G cells, the luciferase activity is induced by the addition of IFN-α and the luciferase activity is expressed at least up to 4 h after IFN-α stimulation (Fig. 2B). Therefore the cloned fragment contains an IFN-responsive element(s). Then plasmids harboring mutated promoters were subjected to this assay (Table 1). The IFN-inducible transcription can be seen with pMxluc1–643, pMxluc450–643, pMxluc499–643, pMxluc531–643, and pMxluc556–643. The plasmid pMxluc586–643 gives neither basal level transcription nor IFN-inducible transcription. Therefore the region between nucleotide positions −59 and −30 upstream of the transcription initiation site contains an IFN-responsive element. Moreover, the basal promoter activity is drastically reduced when the region between nucleotide positions −165 and −117 is deleted. This may be due to the presence of a regulatory sequence(s) in this region involved in activation of transcription.

4. Discussion

This study confirms that the synthesis of *Mx*A mRNA is induced by the addition of IFN. Furthermore it is indicated that the region between nucleotide positions −59 and −30 upstream of the transcription initiation site contains an IFN-responsive element. This element contains the sequence motif somewhat similar to ISRE (Fig. 3). Although this type of motif is also present around the nucleotide position −90, this upstream motif is dispensable for the IFN inducibility. The attachment of IFN-α to the cell surface receptor results in activation of ISGF-3 (interferon-stimulated gene factor-3) which is capable of binding to ISRE and thereby activating transcription [16–19]. Although at present it is not known whether or not ISGF-3 does bind to the IFN-responsive element in the *Mx*A gene, it is likely that the IFN-inducible transcription of the *Mx*A gene is mediated by this transcription factor or its related factors due to the sequence similarity. It is also suggested that the region between nucleotide positions −165 and −117 is involved in efficient basal transcription level.

It is possible that the *Mx*A promoter contains an element responsible for transcription activation by influenza virus infection. The RT-PCR experiment (Fig. 1C) reveals that the synthesis of MxA mRNA is induced after influenza virus infection even in the presence of cycloheximide, an inhibitor of protein synthesis. Moreover, the promoter activity was increased 1.5–2-fold by influenza virus infection in the presence as well as in the absence of IFN in the cells transfected with pMxluc1–643 (data not shown). These observations suggest that the mechanism for induction by virus infection may be independent of that by IFN. Recent studies have reported four virus-inducible elements in IFN-β gene. The IFN-β promoter is activated by virus infection through the element containing PRDI to PRDIV, to which HMGI, ATF-2, IRF and NF-κB bind [20]. However, none of these binding sites are found in the *Mx*A promoter. It is likely that the regulation of the virus-dependent expression of the *Mx*A gene is exerted in different ways.

Acknowledgements: We thank Dr. P. Staeheli for kindly providing cDNAs for MxA and MxB, and K. Hoshida for his support at the initial stage of this work. This work was supported in part by a grant-in-aid from the Ministry of Education, Science, Sports, and Culture of Japan, a grant for the Biodesign Research Program from RIKEN, and a grant from Asahi Glass Foundation.

Table 1
Activity of the human MxA promoter in T98G cells

Plasmid	Exp. 1			Exp. 2		
	+IFN	−IFN	Fold[a]	+IFN	−IFN	Fold[a]
pMxluc1–643	100[b]	3.7	27			
pMxluc450–643	180	4.2	43	100[b]	4.1	24
pMxluc499–643				42	0.6	70
pMxluc531–643				10	0.3	33
pMxluc556–643				12	0.2	60
pMxluc586–643				<0.2	0.2	<1
pSV40luc	46	84	0.55	137	261	0.52

[a]'Fold' gives the ratio of luciferase activity in IFN-treated cells over that in untreated cells.
[b]Results are represented as the values relative to that obtained using pMxluc1–643 and pMxluc450–643 in IFN-treated cells for experiments 1 and 2, respectively.

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