Volume 226, number 2, 297–302 FEB 05454 January 1988

Sequences both 5′ and 3′ to the transcription initiation site contribute to the ability of a mouse H-2 gene to respond to type I interferon

Anna Pascucci, Antonio Pannuti, Girolama La Mantia and Luigi Lania

Dipartimento di Genetica, Biologia Generale e Molecolare, University of Naples, Via Mezzocannone 8, 80134 Naples, Italy

Received 2 November 1987

To investigate the *cis*-acting DNA elements that are involved in the regulation of class I major histocompatibility complex genes by interferon, several promoter fragments of the H-2K^k gene were linked to the reporter chloramphenicol acetyl transferase (CAT) gene, and the CAT expression was analyzed in stable transfected cell lines. The functional activities of progressive deletions of the 5′-flanking region of the H-2K^k gene linked to the CAT gene have allowed us to define a discrete *cis*-acting DNA region necessary for interferon-mediated stimulation. Moreover, the H-2K^k gene transcribed by the nonregulated SV40 early promoter was also found to be under interferon regulation. Thus interferon enhancement of the H-2K^k gene expression appears to be mediated by two *cis*-acting elements, one located in the 5′-flanking region and the other by sequences downstream from the transcription initiation site.

Interferon; H-2 gene transcription; Gene regulation

1. INTRODUCTION

Major histocompatibility complex (MHC) class I genes (termed H-2 K,D and L in mice) encode highly polymorphic membrane antigens that are expressed on nearly all cell types and play an essential role in T-cell immunity [1]. Interferons (IFNs) have been found to stimulate MHC class I gene expression [2–5]. To elucidate the molecular mechanisms of IFN enhancement of the H-2 class I gene expression, several promoter fragments of H-2K^k were linked to the structural gene of chloramphenicol acetyl transferase (CAT) and introduced into rodent fibroblasts. Measurements of CAT activity in IFN-treated cells revealed that sequences from −213 to −165 within the 5′-flanking region of the gene are involved in the stimulation by IFN. Moreover, the promoter-less H-2K^k gene linked to the nonregulated SV40 early promoter was also analysed. The expression of the H-2K^k gene transcribed by the SV40 promoter was shown to be under IFN control.

Thus IFN-mediated enhancement of the H-2K^k gene depends on two discrete *cis*-acting elements, one located in the 5′-flanking and the other downstream from the transcription initiation site.

Correspondence address: L. Lania, Dipartimento di Genetica, Biologia Generale e Molecolare, University of Naples, Via Mezzocannone 8, 80134 Naples, Italy

2. MATERIALS AND METHODS

2.1. *Plasmids*

The pH-2K^k-CAT contains 1.8 kb of the 5′-flanking region of H-2K^k linked to CAT sequences [6]. Fragments containing various lengths of the H-2K^k promoter region were inserted in p8-CAT-0 via *Hin*dIII linkers or using suitable sites present in the promoter-less p8-CAT-0 [7]. The pd1365dS-CAT was isolated after *Sac*II diges-

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

tion of pd1365-CAT and subsequent ligation. The pSV-H-2K^k was constructed in two steps: first, a deletion of the 5′-flanking region of H-2K^k [8] was made using the *Exo*III/Mung-deletion kit as supplied by Stratagene, the resulting promoter-less H-2K^k containing a unique *Eco*RI site at nucleotide +18 relative to the transcription initiation site; second, the *Eco*RI fragment from p861-CAT [7] containing the SV40 early promoter and 256 bp of CAT sequences, was inserted in the *Eco*RI site of the promoter-less H-2K^k. All the plasmids were confirmed by DNA sequencing and isolated by standard recombinant DNA techniques.

2.2. *Isolation of stably transfected cell lines and IFN treatment*

L(TK$^-$) cells were maintained in DMEM (Gibco) supplemented with 5% fetal calf serum. The promoter-CAT plasmids were transfected in L(TK$^-$) cells by the Ca-PO_4 co-transfection technique using the pTK plasmid as selectable marker in HAT medium. The cells were grown and treated with mouse type I IFN (Lee BioMolecular, San Diego, CA) by culturing them in the presence of 1000 U/ml for 48 h. CAT assays were performed as in [9] using 200 μg of total protein extract, protein concentrations were determined using the Bio-Rad protein assay.

2.3. *RNase analysis*

Total cellular RNA was isolated by the guanidinium isothiocyanate method. RNase mapping [10] was performed using uniformly labeled RNA probe transcribed in vitro by T7 polymerase from linear DNA template. The *Xba*I-*Pvu*II fragment from pH-2K^k-CAT, containing 157 bp of CAT sequences and 365 bp of 5′-flanking H-2K^k was cloned in pGEM-3 vector (Promega-Biotec). To analyze the RNA in cells containing the pSV-H-2K^k the 497 bp *Pvu*II fragment of pSV2-CAT [9] containing the SV40 promoter and 157 of CAT sequences was cloned in pGEM-3 (Promega-Biotec) and RNA probe was made. The linearized DNA templates were transcribed in vitro using the reagents and conditions supplied by Promega-Biotec. RNA-RNA hybridization was performed overnight at 45°C. Digestion with RNase A (40 μg/ml) and T_1 (2 μg/ml) was at room temperature for 30 min. The double-strand products were analyzed by electrophoresis through 8% polyacrylamide denaturing gels.

2.4. *Nuclear run-off*

Nuclear run-off transcription assays were performed essentially as in [11–13]. The plasmids were linearized, denatured and spotted onto nitrocellulose filters using the Scheicher & Schull slot-blot apparatus.

3. RESULTS

3.1. *Transfection of the H-2-promoter-CAT into mouse cells*

We constructed a series of H-2K^k promoter-CAT hybrid genes in which the 5′-flanking region of the H-2K^k was deleted progressively from 1.8 kb to 61 bp from the cap site (fig.1). The deletion junctions were sequenced and named with numbers corresponding to the remaining bases of the H-2 promoter with respect to the transcription start site. The gene constructs were cotransfected with Herpes virus tk gene as selectable marker for stable transformation into mouse L(TK$^-$) cells. The majority of stable transformants on each plate (>100) were pooled to minimize the effect of the integration site, copy number and reorganization during the transfection. DNA Southern hybridization, carried out to determine the relative copy number in the isolated clones, showed a range of 1–4 copies of promoter-CAT gene per haploid genome (not shown). The basal levels of CAT gene expression in representative cell cultures of each transfection are reported in fig.1. The basal (unstimulated) levels of the transfected plasmids indicate the presence of two regulatory sequences. One is located between −213 and −165, the other between −99 and −61. These two regions are consistent with the localization of two enhancer sequences designated A and B, respectively, for the H-2K^b [14]. Moreover, sequence comparison between H-2K^b and H-2K^k 5′-flanking regions revealed no difference in these two regions; therefore we conclude that the promoter of H-2K^k contains the two enhancer-like elements A and B as described for the H-2K^b promoter. In addition a 30-bp sequence located at positions −169 to −140 is homologous to a common sequence that has been recently found in the promoter region of several human genes responsive to IFN [15]. This

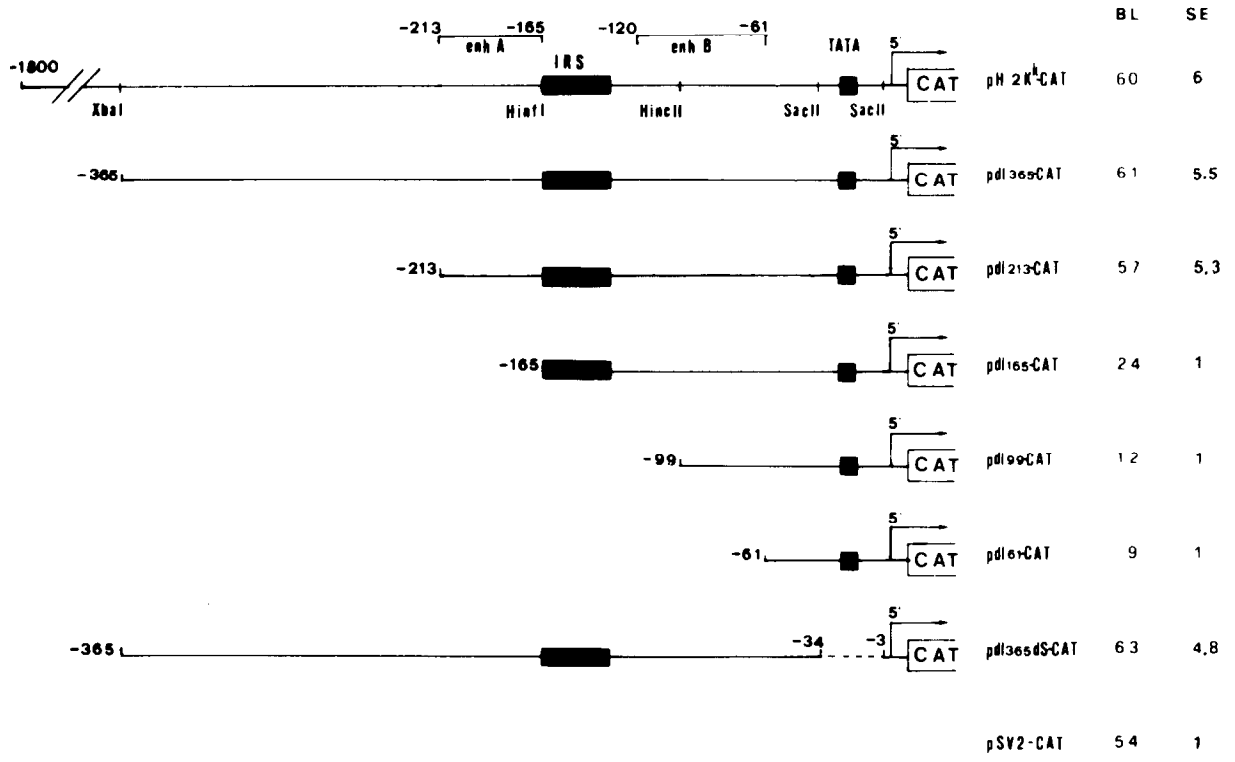

Fig.1. Summary of CAT assays in stable transfected cells, and localization of the IFN-sensitive site in the 5′-flanking region of H-2K^k gene. The deletion constructs are designated by the number of bases remaining relative to the transcription start site. enh A and enh B, the positions of two enhancer sequences as defined [14]; IRS, the IFN-consensus sequences [15]. The basal levels of CAT gene expression varied between different cell lines for each transfected plasmid. The BL values (basal level) reported are the average of at least 3 different cell lines and are expressed as units per mg of protein (1 unit = 1 nmol of chloramphenicol acetylated per h at 37°C). The SE (stimulation effect) was calculated as the average of the ratio of specific CAT activity in cells treated with IFN to that in untreated cells. The measurements of the SE values were made in parallel on the complete set of transfectants, using appropriate amounts of protein to fall within the 10–50% acetylated range.

region has been designated IRS for interferon-responsive sequences.

3.2. *Induction of H-2-CAT expression by type I interferon*

The stable transfected cells were incubated with 1000 U/ml of mouse type I interferon for 48 h. For control, the plasmid pSV2-CAT, which contains the SV40 early promoter, was transfected into L cells. The pSV2-CAT gene expression was not influenced by IFN. The analysis of the CAT activities in the presence and absence of IFN is shown in fig.1. We found that the plasmids pH-2K^k-CAT, pd1365-CAT, pd1213-CAT and pd1365dS-CAT retain the IFN-mediated response. These experiments indicate that sequences from −213 to −165 are important for the IFN response; moreover an internal deletion from position −34 to −3, encompassing the TATA box does not affect IFN stimulation. Our data indicate that the enhancer A is a key element in the IFN response. Next we sought to confirm that the CAT activities measured in the experiments shown in fig.1 reflect initiation of CAT gene transcripts at the correct sites. Total cellular RNA was isolated from cells grown in the presence or absence of type I IFN, and analyzed by RNase mapping [10]. Fig.2 shows the results of such an analysis, indicating that the level of mRNA observed correlates with the levels of CAT activity, and the H-2K^k-CAT mRNA has the appropriate 5′-ends in both the presence or absence of IFN. Under the same conditions, we

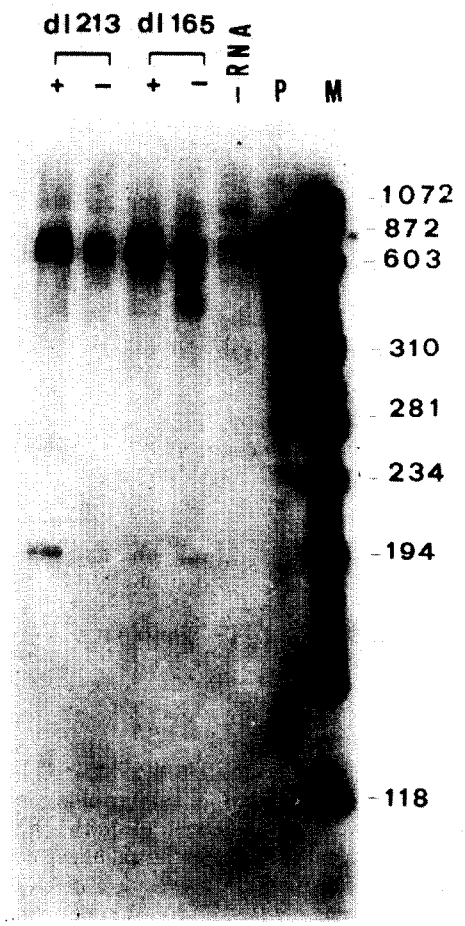

Fig.2. Analysis of transcription initiation sites by RNase protection. Total RNA (20 μg) isolated from stable transformed cells in the presence (+) or absence (−) of IFN was hybridized to ^{32}P-labeled anti-sense CAT RNA. Hybrids were treated with RNase A and T and analyzed on sequencing gels. The pd1165-CAT does not respond to IFN-mediated stimulation (fig.1). The correct transcription initiation start (the protected band of 174 bp) is detected; moreover, aberrant transcription initiation sites were found in the transfected cells. P, untreated anti-sense probe (the 130 nucleotide band is an aberrant SP6 transcript that occasionally was detected, but it did not affect the RNase experiment); −RNA, treated probe in the absence of RNA; M, ^{32}P-labeled *Hae*III digest fragments of ϕX174 DNA.

confirmed by Northern blotting analysis that the steady-state level of endogenous class I genes is stimulated by type I IFN (not shown). From the results in fig.2, the IFN stimulation of H-2 gene expression appears to be due to elevated levels of transcription; however, we cannot exclude the possibility of a post-transcription regulation by type I IFN. We have experimentally addressed this point by measuring the relative rate of transcription of H-2K^k-CAT genes in the presence of IFN, using the nuclear run-off transcription assay [11,12]. An average of 2.5-fold increase in CAT transcription activity was observed in response to type I IFN. Although this increase is small, it was highly reproducible. An example of one such experiment in fig.3 shows that the induction of H-2K-CAT transcripts in IFN-treated cells is at least in part at the level of transcription.

3.3. *Post-transcription regulation of H-2K gene expression by IFN*

The 2-fold increase in transcription is less than the 5-fold enhancement of the CAT activities in the IFN-treated cells (figs 1 and 3). This difference may well be the result of a technical artefact; alternatively, post-transcriptional events such as cytoplasmic stability may also contribute to the enhanced expression of CAT mRNA in IFN-treated cells. To further define this IFN-mediated stimulation of H-2 gene expression, we have con-

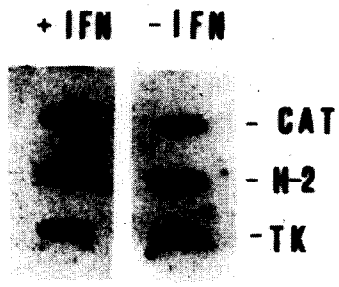

Fig.3. Transcription rate of CAT sequences in transformed cells. Nuclei were isolated from cells transfected with pd1365-CAT with (+) or without (−) IFN treatment, and assayed by nuclear transcription run-off analysis as described in the text. The cloned DNAs bound to nitrocellulose were: CAT, 1.6 kb *Hin*dIII–*Bam*HI fragment from pSV2-CAT; H-2, 1.9 kb *Xba*I–*Xba*I fragment of H-2K^k gene [8], this fragment contains the first 3 exons of the gene; TK, pTK plasmid DNA. Densitometric scanning of the autoradiograph indicates a 2- to 3-fold increase of CAT and H-2 transcription rate relative to that of the TK in IFN-treated cells.

structed a hybrid gene consisting of the SV40 early promoter and the H-2K^k gene [8] lacking its cognate promoter; 157 bp of CAT sequences were located between the SV40 promoter and the H-2 gene in order to discriminate, at the RNA level, the expression of the transfected hybrid gene from the endogenous H-2 genes.

Stable transfected cell lines were isolated after cotransfection into L(TK$^-$) of pSV-H-2K^k hybrid gene with the pTK as selectable marker. All the stable transformants on each plate (>100) were pooled and grown in culture. We then cultured the stable transfected cells in the presence of 1000 U/ml of type I IFN for 48 h, after which total RNA was isolated from cells grown in the presence or absence of IFN, and analyzed by RNase mapping. The result of one such analysis is shown in fig.4. We found that type I IFN is still capable of enhancing the expression of pSV-H-2K^k hybrid gene. Since the SV40 early promoter is not regulated by IFN (see fig.1) we conclude that IFN-mediated enhancement of H-2K^k gene depends on two different mechanisms; one is controlled by the 5′-flanking region and the other by sequences downstream from the transcription site.

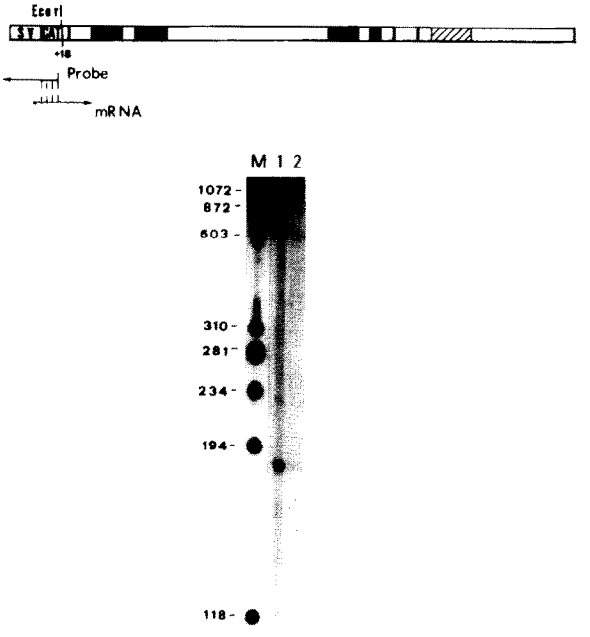

Fig.4. DNA sequences 3′ from the transcription initiation site contribute to the ability of H-2K^k to respond to type I IFN. On the top is shown the structure of the pSV-H-2K^k. Filled boxes denote exons and open boxes denote introns and flanking sequences. The dashed box shows the 3′ untranslated region. SV, *Pvu*II-*Hin*dIII fragment of SV-40 containing the early promoter; CAT, 157 bp *Hin*dIII-*Pvu*II CAT fragment. Cells transfected with pSV-H-2K^k were treated with IFN and analyzed by RNase protection using an anti-sense probe. The RNase protection was performed as described in the text. The correct transcription initiation start (the protected band of 174 bp) was detected. 1, RNA isolated from IFN-treated cells; 2, the IFN-untreated sample; M, ^{32}P-labeled *Hae*III DNA fragment of ϕX174.

4. DISCUSSION

We found that the region from position −213 to −165 relative to the mRNA cap site of the H-2K^k is important for IFN regulation. This region includes the enhancer-like sequences A and overlaps the 30 bp IRS consensus sequences [15], located between −169 and −140. An internal deletion from nt.-34 to nt.-3, encompassing the TATA box, does not affect the IFN response. Our results are comparable and consistent with those obtained by others with H-2K^b and H-2D^d genes [16–18], suggesting the presence of similar regulatory sequences in distinct class I genes. Using the nuclear run-off analysis we have shown that the IFN-response is at least in part at the level of transcription. However, we have also found that IFN-mediated increase of H-2K^k gene expression is controlled by sequences located 3′ to the transcription initiation site. This result extends the original observation [19] that the expression of a promoterless human class I gene HLA-B7, transfected into mouse L cells is regulated by IFN. Similar results on the localization of 3′IFN regulatory sequences have been described recently for the H-2L^d and H-2D^d gene [20].

The presence of two different regions in the H-2K gene involved in IFN mediated activation suggests the existence of at least two different mechanisms, which appear to act independently of each other, and account together for the full response of H-2K^k gene expression to type I IFN. One of the mechanisms involving sequences at the 5′-flanking region, clearly operates at the level of

initiation of gene transcription. The molecular mechanism, involving downstream regulatory sequences remains undetermined. Although the localization and identity of the 3′ regulatory sequences have not been established, it may be that these regions are involved in post-transcription events such as an increase in RNA stability.

ACKNOWLEDGEMENTS

We thank R. Terracciano for technical assistance and M. Sbordone for typing the manuscript. A. Pascucci is supported by a fellowship from Associazione Italiana per la Ricerca sul Cancro. This work was supported by grants from Progetti Finalizzati CNR PF 'Oncologia' and 'Ingegneria Genetica' and from AIRC to L.L.

REFERENCES

[1] Hood, L., Steinmetz, M. and Malissen, B. (1983) Annu. Rev. Immunol. 1, 529–568.
[2] Fellous, M., Nir, U., Wallach, D., Merlin, G., Rubinstein, M. and Revel, M. (1982) Proc. Natl. Acad. Sci. USA 79, 3082–3086.
[3] Burrone, O.R. and Milstein, C. (1982) EMBO J. 1, 345–349.
[4] Friedmann, R.L., Mainly, S.P., McMahon, M., Kerr, I.M. and Stark, G.R. (1984) Cell 38, 745–755.
[5] Revel, M. and Chebath, J. (1986) TIBS 11, 166–170.
[6] Pannuti, A., La Mantia, G. and Lania, L. (1987) Nucleic Acids Res. 15, 1595–1631.
[7] Pannuti, A., Pascucci, A., La Mantia, G., Fischer-Fantuzzi, L., Vesco, C. and Lania, L. (1987) J. Virol. 61, 1296–1299.
[8] Arnold, B., Burget, H.G., Archibald, A.L. and Kvist, S. (1984) Nucleic Acids Res. 12, 9473–9487.
[9] Gorman, C.M., Moffat, L.F. and Howard, B.H. (1982) Mol. Cell Biol. 2, 1044–1051.
[10] Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K. and Green, M.R. (1984) Nucleic Acids Res. 12, 7035–7056.
[11] Groudine, M., Peretz, M. and Weintraub, H. (1981) Mol. Cell Biol. 1, 281–288.
[12] Greenberg, M.E. and Ziff, E.B. (1984) Nature 311, 433–438.
[13] Lania, L., Pannuti, A., La Mantia, G. and Basilico, C. (1987) FEBS Lett. 219, 400–404.
[14] Kimura, A., Israel, A., Le Bail, O. and Kourilsky, P. (1986) Cell 44, 261–272.
[15] Friedmann, R.L. and Stark, G.R. (1985) Nature 311, 433–438.
[16] Israel, A., Kimura, A., Fournier, A., Fellous, M. and Kourilsky, P. (1986) Nature 322, 743–746.
[17] Vogel, J., Kress, M., Khoury, G. and Jay, G. (1986) Mol. Cell Biol. 6, 3550–3554.
[18] Baldwin, A.S. and Sharp, P.A. (1987) Mol. Cell Biol. 7, 305–313.
[19] Yoshie, O., Schmidt, H., Lengyel, P., Reddy, E.S.P., Margan, W.R. and Weissman, S.M. (1984) Proc. Natl. Acad. Sci. USA 81, 649–653.
[20] Korber, B., Hood, L. and Strynowsky, I. (1987) Proc. Natl. Acad. Sci. USA 84, 3380–3384.