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Two distinct pathways of interferon induction as revealed by 2-aminopurine

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ABSTRACT

Activation and repression of IFN gene expression are controlled primarily at the transcriptional level. In order to elucidate some aspects of the induction mechanism of the IFN genes, we examined the effects of different treatments on IFN production in L929 cells, a well-characterized system, and in primary spleen cells. Our results indicate that 2-Aminopurine (2-AP) inhibits type I IFN (IFN-α and IFN-β) induction in L929 cells but not in spleen cells. In L929 cells, 2-AP inhibited the induction of the MuIFN-β promoter and of promoters containing tetrahexamer and PRDII sequences linked to a reporter gene. Inhibition of activation of the inducible factors binding to the MuIFN-β promoter and sub-elements was also observed. In contrast, factors binding to the MuIFN-β promoter are present constitutively in spleen cell nuclei and their activity is not inhibited by 2-AP. These results suggest that 2-AP inhibits IFN-β gene induction in L929 cells through blocking of activation of the inducible DNA-binding factors which interact with the IFN-β promoter.

INTRODUCTION

Expression of the type I interferon (IFN) genes (IFN-α and IFN-β) is induced by virus infection or treatment of cells with double-stranded RNA (dsRNA). The molecular signal for the viral induction of Type I IFNs appears to be the dsRNA generated during infection (for reviews, see 1). IFN treatment is known to induce the expression of a dsRNA-dependent protein kinase and it has been proposed that this enzyme is also involved in induction of the IFN genes themselves (2,3). Although the details of the mechanism of induction is not clear, DNA sequences required for induction have been extensively characterized, especially in the case of the HuIFN-β gene(4–8). The regulatory element of this gene consists of three positive regulatory domains (PRD), and two negative regulatory domains (NRD) which have been defined by mutational analysis of the promoter(4–9). Different DNA-binding factors recognize the PRDs and cDNAs corresponding to some of these have been cloned (10–17).

We have reported previously that in spleen cells of inbred mice, no significant changes in levels of activities binding to regulatory elements of the IFN-β gene were observed when transcription was induced (18). This is in contrast to other systems such as L929 fibroblasts.

To further characterize the mechanism of induction of IFN genes in spleen cells and L929 fibroblasts, we treated both cell types with drugs, in particular 2-Aminopurine (2-AP) a protein kinase inhibitor which has been reported to inhibit IFN induction (2,3). We examined the effect of this inhibitor on the activity of regulatory elements of the IFN-β gene and on the levels of factors which bind to these elements.

Our results indicate that 2-AP inhibits the induction of both IFN-α and IFN-β genes in L929 cells, but not in spleen cells. Treatment with 2-AP decreased transcription dependent on both PRDI (TH) and PRDII elements and blocked induction of activities binding to these elements.

MATERIALS AND METHODS

Animals, cell culture, virus induction and IFN titrations

Spleen cell suspensions were prepared from male mice of C57BL/6 (Charles River Canada Inc.) and L929 cells were cultured as previously described (19). Cell monolayers and spleen cell suspensions were infected with NDV (50 to 100 PFU/cell) for 2 hrs at 37°C. The virus was removed and replaced with serum-free minimal essential medium. IFN titrations were carried out by the method of cytopathic effect reduction in L929 cells infected with encephelomyocarditis virus as previously described (20).

Isolation and analysis of RNA

Total RNA was isolated by the method of Murphy (21) and was passaged twice through oligo (dt) cellulose (type 7, Pharmacia) and Northern blotting was done as described by Sambrook et al (22). Probes pGEMIFNβ, pGEMIFNα (as previously described, 19) and pGEM IRF-1 (23) were labeled with [^{32}P] αCTP by nick-translation (22).

Transient expression assays

L929 cells were co-transfected with 10 μg of CsCl-purified chloramphenicol acetyl-transferase (CAT) reporter plasmid DNA

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and 10 μg of CsCl-purified β-galactosidase (β-GAL) control plasmid DNA (RSV-β-GAL). Transfections were carried out by the calcium phosphate coprecipitation method (20). In spleen cells, transfections were carried out by electroporation as described previously (18).

Cells were induced 24 to 26 hours post-transfection and harvested 15 hours post-induction. The CAT and β-GAL assays were done as described by Sambrook et al (22) and the percentage of acetylation was normalized to the β-GAL activities.

The −294 to +8 sequence of the MuIFN-β promoter was obtained by polymerase chain reaction amplification (22) of mouse genomic DNA. The clone obtained was sequenced and found to be identical to the sequence described by Dirks and Hauser (24). This sequence was removed, ends were made blunt with Klenow fragment (22) and it was ligated into SphI-AccI-cleaved pSV$_2$CAT vector (25). TH-CAT (Tetra hexamer) and P2-CAT (PRDII) were provided by J.Hiscott (9).

Preparation of nuclear extracts

Nuclear extracts were prepared essentially by the method of Dignam et al. (26). After virus induction, the cells were resuspended in 2 packed cell volumes (PCV) of 25 mM Tris-HCl (pH 7,5; 25 mM KCl; 7,5 mM $MgCl_2$; 0,2 mM phenylmethyl sulfonyl fluoride (PMSF); 30% sucrose; and 0,01% Nonidet P-40 (NP-40) and incubated on ice (15 minutes). The cells were pelleted in the cold and the nuclear pellet was resuspended in 0,1 PCV of buffer (25% glycerol; 400 mM NaCl; 20 mM Hepes pH 7,9; 1,5 mM $MgCl_2$; 0,2 mM EDTA; 0,5 mM DTT; 0,5 mM PMSF; 100 u/ml of aprotinin and 5 μg/μl of leupeptin). Nuclei were gently resuspended every 10 minutes on ice for 30 minutes. At the end of this time, nuclei were centrifuged for 15 minutes at 1800g and the supernatant fraction was dialysed overnight against 1000 ml of 20% glycerol; 20 mM Hepes pH 7,9; 100 mM KCl; 0,2 mM EDTA; 0,5 mM DTT; 0,5 mM PMSF; 20 u/ml of aprotinin and 0,5 μg/ml of leupeptin. After dialysis, extracts were clarified for 20 minutes at 1800 g, then aliquoted and stored in liquid nitrogen. Protein determinations were done using the Bio-Rad protein assay kit (Bio-Rad, Missisauga, Ontario, Canada).

Gel retardation assays

The gel retardation assay was performed as previously described (27) with the following modifications. Reactions contained: nuclear extract (2,5 to 5 μg); 2,5 μg of poly(dI).poly(dC) as non-specific competitor, 25mM Hepes (pH 7,9), 1 mM DTT, 40 mM NaCl, 1 mM EDTA and 5% glycerol (for a total volume of 25 μl). After 20 minutes at 25°C, the probe (5,000 to 10,000 cpm) was added and the reaction was incubated for another 20 minutes at 25°C. At this point 3μl of Blue II (22) sample buffer was added and the samples were electrophoresed in 5% polyacrylamide gels containing 50 mM Tris pH 7,5; 380 mM glycine, 2 mM EDTA. The gels were run at 150 v for 1,5−2,5 h, after which they were dried and exposed to autoradiographic film with intensifying screens at −70°C.

Methylation interference assays

The murine IFN-β promoter DNA (−130 to −37) was labeled using (γ-^{32}P) ATP (ICN, 3888 Ci/mmol) and methylated with dimethyl sulfate (28). Approximatively 2×10^5 cpm of the labeled DNA was used in gel retardation assays. After autoradiography the bands containing different complexes were excised from the gels. The DNA was electroeluted and cleaved with NaOH as described by Maxam and Gilbert (28), phenol/chloroform extracted, ethanol-precipitated, then washed with 70% ethanol and air-dried. The radioactive sample was separated on 6% sequencing gels.

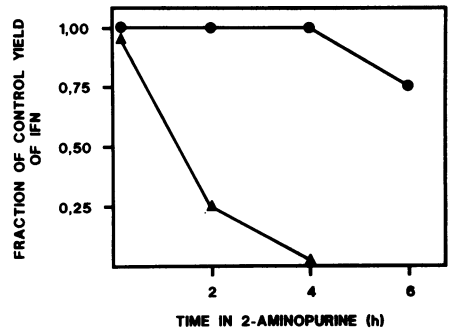


Figure 1. Time course of inhibition of IFN yield by 2-AP. L929 cells (triangles) and spleen cells (circles) were induced in the presence of 2-AP, kept in for the time interval shown on the abscissa, and then washed out. Fresh medium was added and incubated for a total of 10 h. Control yield of IFN for L929 cells and spleen cells were 760 u/ml and 384 u/ml respectively.

Table 1. Effect of different treatments on IFN titers[a] in L929 cells and spleen cells.

Treatment	L929 cells		spleen cells	
	Mock-induced	induced	Mock-induced	induced
cycloheximide[b]	<6	768	<6	384
IFN-(α+β)	<6	768	<6	384
anti-IFN(α+β)	<6	768	<6	384
2-AP[c]	<6	192	<6	384
none (mock)	<6	768	<6	384

[a] IFN titers were determined 10 h post-induction and all titers are in laboratory units / ml. In this system 1 U corresponds to 4 IU as measured by reference MuIFN preparation G-002-904-511.
[b] Cells were incubated with cycloheximide (100 μg/ml),IFN-(α+β) (1000 u/ml) or anti-IFN-(α+β) (1000 u/ml) for 6 h and during the last 2 h, virus was added.
[c] Cells were induced for 2 h in the presence of 10 mM 2-Aminopurine.

RESULTS

Treatment of spleen and L929 cells with different drugs

We determined the effect of treatment of L929 cells and spleen cells with the protein synthesis inhibitor cycloheximide; IFN (αβ); antibody to IFN-αβ and the kinase inhibitor, 2-Aminopurine (2-AP) (Table 1) on IFN induction. Of all of these treatments, only 2-AP was found to decrease IFN yield in L929 cells. This result is similar to that obtained by Marcus et al. (2) and Zinn et al (3). None of the treatments, including 2-AP, had a significant effect on induction of spleen cells. We observed the same results with spleen cells from other inbred mouse strain (i.e. BALB/c; data not shown).

If we compare the effect of 2-AP treatment on induction of L929 cells and spleen cells (Fig. 1), we observe that in the former, the presence of the inhibitor during the first two hours of induction leads to a 75% reduction in IFN titre and a four hour treatment leads to a 95% decrease. In spleen cells, even a 6 hour treatment of spleen cells only causes a 25% decrease in IFN titre.

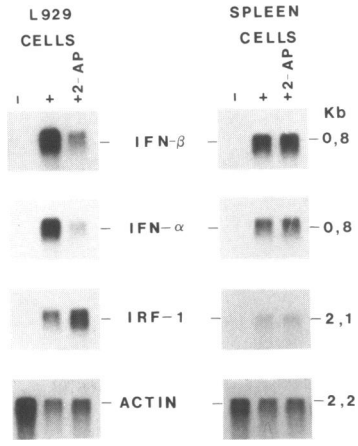


Figure 2. Northern blot analysis of poly $(A)^+$ RNA from L929 cells and spleen cells treated for 2 h with 2-AP during the induction. Poly $(A)^+$ RNA (1 μg) from mock-induced (−), induced (+, 8 h for L929 cells and 3 h for spleen cells) and induced in the presence of 2-AP (+2-AP, for a total of 8 h for L929 cells and 3 h for spleen cells) were hybridized to the ^{32}P-labeled MuIFN-β cDNA (18). After autoradiography, the probe was eluted and the filter was rehybridized consecutively with MuIFN-α cDNA (18), MuIRF-1 (22) and chicken β-actin.

Table 2. IFN production in L929 cells and spleen cells in the presence of 2-Aminopurine during the induction.

Induction 2-AP		NONE	anti IFN-(α+β)[a]	anti IFN-β
L929	− −	<6	−	−
cells	+ −	768	<6	192
	+ +	192	<6	48
spleen	− −	<6	−	−
cells	+ −	384	<6	48
	+ +	384	<6	12

[a] 500 u of rabbit antisera (Lee, Biomolecular Research Lab. Inc.) for murine IFN-(α+β) and murine IFN-β were incubated at 4°C during 4 h.

Neutralization with antibodies specific for IFN-αβ or IFN-β alone shows that induction of both IFN-α and IFN-β were inhibited in L929 cells by the presence of 2-AP (Table 2).

Levels of IFN-specific mRNA in cells induced in the presence of 2-AP

Fig. 2 shows Northern blots of poly (A)+ RNA from L929 cells and spleen cells induced with NDV in the presence or absence of 10 mM 2-AP. As expected, in L929 cells, 2-AP inhibits the induction of both IFN-β and IFN-α mRNA. The induction of other virus-inducible genes, interferon regulatory factor 1 (IRF-1; Fig. 2) and Tissue Inhibitor of Metalloproteinases (TIMP, data not shown, ref.29) was not affected by the presence of 2-AP during the induction. In spleen cells, 2-AP had no effect on induction of any of the mRNAs tested (Fig. 2).

2-Aminopurine inhibits the induction of the MuIFN-β promoter and of PRDI-like (Tetra hexamer) and PRDII subelements

To further characterize the mechanism of inhibition of IFN induction by 2-AP in L929 cells, we subcloned the MuIFN-β promoter into pSV_2CAT and transfected this construct (MuIFN-β-CAT) into L929 cells. CAT activities were determined before and after virus induction and after induction in the presence of 2-AP (Fig. 3). The relative induction of CAT activites was 12,5-fold after virus infection. This was reduced to 2,6-fold (a 5-fold reduction) when 2-AP was present during the induction. In contrast, 2-AP had no effect on the induction of the MuIFN-β-CAT in spleen cells. The inhibition of the induction of CAT activity by 2-AP in L929 cells correlates well with the inhibition of induction of endogenous IFN mRNAs and activity.

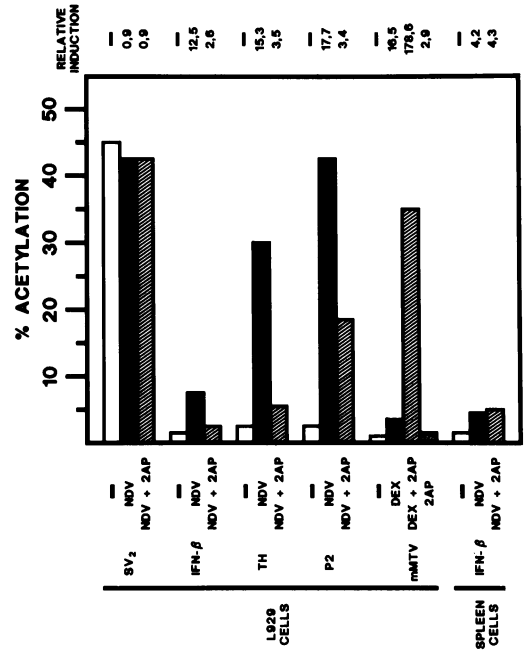


Figure 3. Effect of 2-AP on induction of expression of IFN-CAT, MMTV-CAT in L929 cells and IFN-CAT in spleen cells. L929 cells were transfected and induced for 2 h for the virus induction as described in Materials and Methods and for 4 h with dexamethasone (Dex) at the concentration of 50 μM. Transfected plasmids have been described in Materials and Methods. The bar graphs show the percentage of acetylated chloramphenicol in extracts from mock-induced cells (open bars), induced cells (solid bars) and induced cells in the presence of 2-AP (hatched bars). The percentage of acetylation was normalized to the β-GAL activities. Relative induction was obtained by dividing percent conversion in the induced sample by that of the mock-induced sample.

To determine whether inhibition by 2-AP was specific to IFN-β induction, we examined the effect of the compound on the dexamethasone induction (Dex) of the mouse mammary tumor virus promoter linked to the CAT gene (MMTV-CAT). The relative induction of CAT activities was 16,5 fold after the addition of the dexamethasone. 2-AP increased this induction to a total of 178 fold. 2-AP alone also induced CAT activities (3 fold induction) from MMTV-CAT when used alone (Fig.3). A similar effect of the 2-AP was also observed previously by Zinn et al. (3) with the metallothionein MT-1 gene in mouse NIH 3T3 cells induced with dexamethasone. CAT activities of the SV40 construct were not affected by virus induction and 2-AP.

In L929 cells, expression of CAT from constructs containing PRDI-like (TH-CAT) or PRDII elements (P2-CAT) was also inhibited by 2-AP (a 4 to 5-fold reduction). This suggested that the levels of factors binding to these elements might be affected by this inhibitor of protein kinase activity.

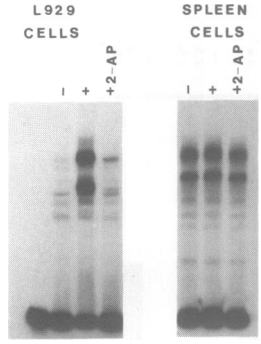
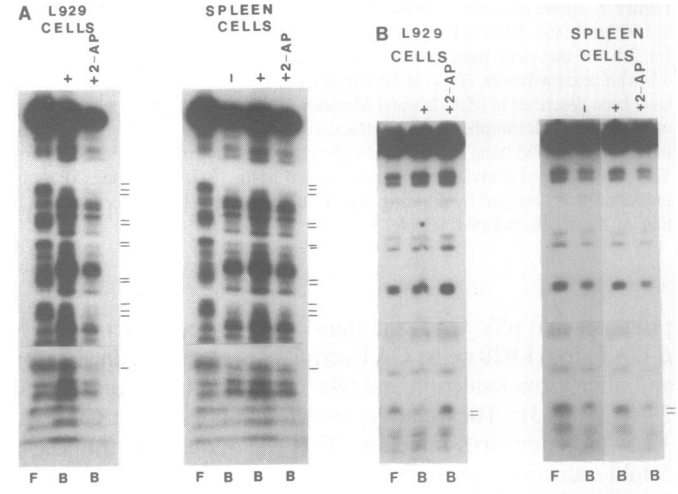


Figure 4. Gel retardation using the MuIFN-β promoter (−134 to −37). The ^{32}P-labeled DNA fragment (Ssp I-Ava II) from the IFN-β promoter was incubated with 2,5 μg of L929 cell nuclear extract from mock-induced, induced (8 h) and induced in the presence of 2-AP (2 h during the induction, washed out followed incubate for an additionnal 6 h), or with 5 μg of spleen cell nuclear extracts from cell mock-induced, induced (3 h) and induced in the presence of 2-AP (2 h during the infection, washed out and incubated for an additionnal 1 h).



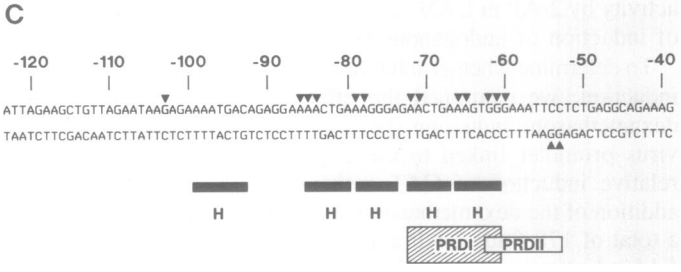


Figure 5. Methylation interference analysis of protein in L929 cell and spleen cell nuclear extracts (protein binding to the MuIFN-β promoter (−134 to −37)). The methylated Ssp I-Ava II fragment (see Materials and Methods) was end-labeled (A for the coding strand and B for the non-coding strand) and incubated with nuclear extracts (see Fig. 4). Bound (B) and free (F) complexes were eluted from a gels following standard DNA-binding reaction. The DNA was treated with NaOH and analysed on 6% poly-acrylamide. (C) Triangles indicate the interfering position on the IFN-β promoter. PRDI, PRDII and HEX motif were assigned by the homology with the HuIFN-β promoter.

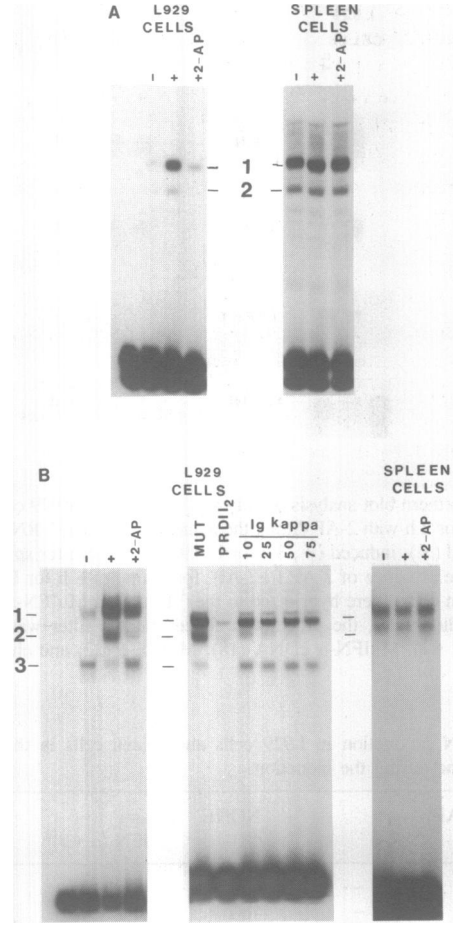


Figure 6. Nuclear factors binding to TH (A) and PRDII$_2$ (B). Nuclear extracts from L929 cells and spleen cells as described in Fig. 4 were incubated with the TH oligonucleotide (AAGTGA$_4$) and PRDII$_2$ oligonucleotide. In (B), different competitors (50 ng) or increasing amounts of competitors as indicated above the gel were included in the gel retardation experiment. Competitors were MUT (5′-TGCGGATTCCCA-3′) which is not recognized by NF-κB,the PRDII$_2$ probe itself and Ig Kappa (5′-AGGGGACTTTCC-3′).

Nuclear factors binding to the IFN-β promoter and its sub-elements were affected by the presence of 2-AP during the induction

Several DNA-binding factors (IRF-1, IRF-2, NK-κB, etc.) have been implicated in the inducible expression of the IFN-β promoter (11−15). In order to determine if some of them were affected by the 2-AP treatment, we carried out gel retardation experiments with the IFN-β promoter (−134 to −37) as probe. When L929 cells were exposed to virus, levels of complexes obtained with this probe were increased. This increase was strongly inhibited by the presence of 2-AP during the induction (Fig.4). We have reported previously that factors binding to the IFN-β promoter in spleen cell nuclear extracts were present constitutively (18). Their levels were not affected by treatment with 2-AP.

Comparison of the methylation interference patterns of the virus-induced complexes and of complexes obtained when induction was carried out in the presence of 2-AP in L929 cells were closely similar (Fig. 5) . Methylation interference patterns observed with nuclear extracts of spleen cells under different

conditions were also essentially identical to those obtained with L929 cell nuclear extracts. This result indicates that the factors binding to the IFN-β promoter exhibited the same close base contacts in nuclei of L929 cells induced in the presence or absence of 2-AP and in spleen cells (Fig. 5).

In L929 cells 2-AP also blocked induction of factors binding to sub-elements of the IFN-β promoter: TH (PRDI-like) and PRDII (Fig.6). Two complexes were detected with the TH probe (Fig. 6A). Both of these complexes were induced by virus and this induction was strongly reduced when 2-AP was present. We have shown previously (18) that the complex 2 comigrated with IRF-1 protein prepared *in vitro*. The other complex may represent IRF-2 or others proteins not yet fully characterized. In the same way, three complexes were detected with the PRDII probe (Fig. 6B). Levels of complexes 1 and 2 were increased after virus induction, but only the levels of complex 2 which we have found to be indistinguishable from NF-κB (18) were affected by the presence of 2-AP. In spleen cell nuclear extracts the presence of 2-AP during the induction had no effect on levels of binding to either the TH or PRDII probes.

DISCUSSION

In the present report, we have compared two different pathways of IFN induction, one which is sensitive to inhibition by 2-AP (L929 cells) and one which is not (spleen cells). The inhibition of IFN induction by 2-AP observed in L929 cells was previously described by Marcus and Sekellick (2). Zinn et al. (3) have reported that 2-AP inhibits IFN-β induction in Human MG63 osteosarcoma cells and they observed that this was due to decreased transcription of the IFN-β gene in the presence of 2-AP. 2-AP inhibits the expression of both IFN-α and IFN-β mRNA in L929 cells (Fig.2). In contrast, virus induction of IRF-1 mRNA (Fig. 2) and TIMP mRNA (data not shown) are not inhibited by 2-AP treatment. This result indicates that there is a commonality or an overlap between the IFN-α and IFN-β virus inducible pathways, which is not shared by other virus inducible genes such as IRF-1 in L929 cells.

Differents groups (30, 31) have previously described the effect of 2-AP effects on gene induction in response to dsRNA and IFN. Some genes (561 and 6−16) which respond to IFN-α and IFN-γ can also be induced directly by dsRNA (30, 31). 2-AP can selectively block the induction of genes 561 and 6−16 by dsRNA but not by IFN-α (30, 31). The results presented here reinforce the concept that the process involved in IFN gene induction and IFN-inducible gene expression are partially overlapping.

In spleen cells, the induction of IFN activity (Fig.1) and the expression of IFN-α, IFN-β and IRF-1 mRNA (Fig.2) are all resistant to 2-AP. The decrease in IFN titre observed after 6 h of treatment by 2-AP could be explained by toxicity since treatment for periods longer than 12 h killed more than 50% of the spleen cells. Control experiments where cells were treated with a carrier solution had no effect on any of the cells tested (data not shown).

In L929 cells, we observed that 2-AP inhibits the virus-inducibility of chimeric genes containing the coding region of the bacterial chloramphenicol acetyl transferase (CAT) and the IFN-β promoter or its sub-elements (PRDI-like and PRDII, Fig.3). These results indicate that the virus-induction of the chimeric genes and the endogenous IFN-β gene are probably inhibited by the 2-AP through the same mechanism. In spleen cells, 2-AP had no effect on the induction either of the MuIFN-β-CAT or the endogenous IFN-β gene.

Enhancement of expression of exogenous genes by 2-AP has been previously reported in L929 stable transfectants (32) but should not be involved in this case since transfections were done transiently and the duration of 2-AP treatments was much shorter (2 h rather than 15 h).

Several DNA-binding factors which recognize sequences within the IFN-β promoter have been characterized (10−17). Many of these bind specifically to PRDI or TH (IRF-1, IRF-2, PRDI-BFi) or to PRDII (PRDII-BF and NF-κB). In gel retardation experiments using these probes, induction of several complexes was inhibited by 2-AP in L929 cells (fig.6 A). In the case of IRF-1 (which binds to the TH probe and corresponds to complex 2), Watanabe et al. (33) have proposed that the active form may be phosphorylated. Our results are in agreement with this possibility since, during induction, 2-AP inhibits induction of TH binding activity (fig.6A) but not the accumulation of IRF-1 mRNA (Fig. 2A). In addition to this, cycloheximide had no effect on induction of IFN (table 1 and ref.34) indicating that *de novo* synthesis of IRF-1 is not essential. Several groups have reported that the inducible PRDII-binding factor is the transcription factor NF-κB (13−15). This factor is present constitutively in B-lymphocytes (36) which constitute the major component in the mixed population of spleen cells. In other cells, this factor is sequestered in the cytoplasm by IκB (inhibitory κB) and is translocated into the nucleus after induction by a variety of factors (PMS, LPS, dsRNA, virus) (34−37). The molecular events leading to the dissociation between NF-κB and IκB seem to involve phosphorylation of IκB (36). The specific inducible PRDII binding complex II is indistinguisable from NF-κB and its induction was also inhibited by the presence of 2-AP (Fig. 6B). These results suggested that the inhibitory effect of the 2-AP on IFN induction was due to the inhibition of the phosphorylation of IRF-1 and IκB.

As we have reported previously (18), in spleen cell nuclear extracts, the complexes observed with the MuIFN-β promoter (Ssp1-AvaII fragment), the TH element and with $PRDII_2$ element were present constitutively and all factors detected with these elements were also found in L929 cell nuclear extracts. We now find that their levels are not inhibited by 2-AP. The failure to detect differences between induced and uninduced cells is not caused by a low percentage of IFN-β producing spleen cells since *in situ* hybridization indicates that over 40% of the cells are positive for IFN-β mRNA (19). Furthermore, the lack of inhibition of IFN induction by 2-AP in spleen cells correlates well with the lack of inhibition of DNA-binding activities specific for IFN promoter elements.

Our results suggest that the mechanism by which 2-AP inhibits IFN-β gene induction in L929 cells is through the inhibition of the induction of IFN-β promoter-binding factors. Furthermore, in spleen cells these DNA-binding factors were present constitutively and were not inhibited by 2-AP. This clearly indicates that at least two different pathways of IFN induction exist. 2-AP is considered to be a specific inhibitor of the IFN-inducible dsRNA dependant protein kinase (3). Some authors have suggested that this kinase, which binds with high affinity to dsRNA (38) is involved in the transcriptional activation of the IFN genes in some cell types (2,3). This would not seem to be the case in spleen cells, where necessary positive factors may already be fully activated. However, elucidation of the exact role of this kinase in IFN induction will require further experiments.

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