The EMBO Journal Vol.17 No.23 pp.6963–6971, 1998

Ser727-dependent recruitment of MCM5 by Stat1α in IFN-γ-induced transcriptional activation

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Stat1α is a latent cytoplasmic transcription factor activated in response to interferon-γ (IFN-γ). The C-terminal 38 amino acids of Stat1α are required to trigger transcription and therefore may possibly serve as a transcription activation domain (TAD). Here we show that the C-terminus of Stat1α is an independent TAD which can interact with a specific group of nuclear proteins. Mutation of the Stat1 Ser727 and Leu724 decreases its transcriptional activity and affinity for the nuclear proteins. One of the interacting proteins was identified as MCM5, a member of the mini-chromosome maintenance (MCM) family involved in DNA replication. Both *in vitro* and *in vivo* interaction of Stat1α and MCM5 were demonstrated. Furthermore, the *in vitro* interaction required Ser727 and was enhanced by its phosphorylation. Transient over-expression of MCM5 enhanced transcriptional activation by Stat1α in a Ser727-dependent manner. Finally, changes in the level of nuclear localized MCM5 during the cell cycle correlated with the changes in transcriptional response to IFN-γ acting through Stat1α. These results strongly suggest that MCM5 is recruited through interaction with Stat1α in a Ser727- and Leu724-dependent manner to play a role in optimal transcriptional activation.

Keywords: interferon/MCM5/Stat1/transcription

Introduction

In response to extracellular ligand binding to cell surface receptors, the signal transducer and activator of transcription proteins (STATs) become phosphorylated on tyrosine, dimerize and enter the nucleus to activate transcription (Leaman *et al*., 1996; Darnell, 1997). The seven currently known mammalian STATs all have crucial physiological roles (Darnell, 1997; O'Shea, 1997) and all are activated by one or more known cytokines (Darnell *et al*., 1994; Schindler and Darnell, 1995). Interferon-γ (IFN-γ) induces phosphorylation of Stat1, which enters the nucleus as a homodimer and binds to DNA elements called IFN-γ-activated sites (GAS) (Decker *et al*., 1991; Lew *et al*., 1991; Shuai *et al*., 1992, 1994). The transcriptional activation domain (TAD) of Stat1α was originally defined by a differential splicing event which leaves out the C-terminal 38 amino acids of Stat1α, the active transcription factor, to produce Stat1β, a transcriptionally inactive form of Stat1 (Schindler *et al*., 1992; Muller *et al*., 1993). To achieve maximal transcriptional activity for Stat1α, a serine residue at position 727 in the C-terminus must also be phosphorylated (Wen *et al*., 1995).

Most DNA-binding transcriptional activators act in conjunction with other proteins, both DNA binding and non-DNA binding (Bjorklund and Kim, 1996; Kaiser and Meisterernst, 1996; Verrijzer and Tjian, 1996). Interactions of STATs have been described with members of the IFN regulatory factor (IRF) family, the steroid and Jun family, and possibly specificity protein-1 (SP1) (Darnell, 1997). In addition, Stat1 and Stat2 have been reported to interact with CREB-binding protein (CBP)/p300 (Bhattacharya *et al*., 1996; Zhang *et al*., 1996; Horvai *et al*., 1997), the histone acetyltransferases involved in chromatin remodeling (Kadonaga, 1998).

In this report we show that a group of nuclear proteins specifically interact with the C-terminus of Stat1α. One of these newly identified proteins is MCM5 (Hu *et al*., 1993; Paul *et al*., 1996), a member of a group of proteins identified first in yeast and later in human cells that increase in their association with chromatin in late G_1 in order to assist in the initiation of DNA replication (Stillman, 1996; Dutta and Bell, 1997; Newlon, 1997). The mini-chromosome maintenance proteins (MCMs) form complexes of various constitutions (Dutta and Bell, 1997; Richter and Knippers, 1997; Holthoff *et al*., 1998), assemble at the initiation site of DNA replication along with other protein complexes such as origin-recognition complex (ORC; Baker and Bell, 1998) and travel together with the replisome to, perhaps, furnish a helicase function (Aparicio *et al*., 1997; Ishimi, 1997; Tanaka *et al*., 1997). We found clear evidence of specific MCM5 interaction with the Stat1α C-terminal domain *in vitro* which is enhanced by phosphoserine-727 and *in vivo* interaction is demonstrated as well. Transient over-expression of MCM5 enhanced transcriptional activation by Stat1α in a Ser727-dependent manner. Finally, we show that the increase in the level of nuclear localized MCM5 in late G_1 is accompanied by increased transcriptional response to IFN-γ. Thus MCM5 is recruited by Stat1 to serve a role in transcriptional activation in addition to its previously identified role in DNA replication.

Results

The C-terminal region of Stat1α is an independent TAD, and Ser727 and Leu724 are required for maximal transcription activation by Stat1α

To analyze further the requirement of Ser727, as well as the conserved Leu724 in transcriptional activation by

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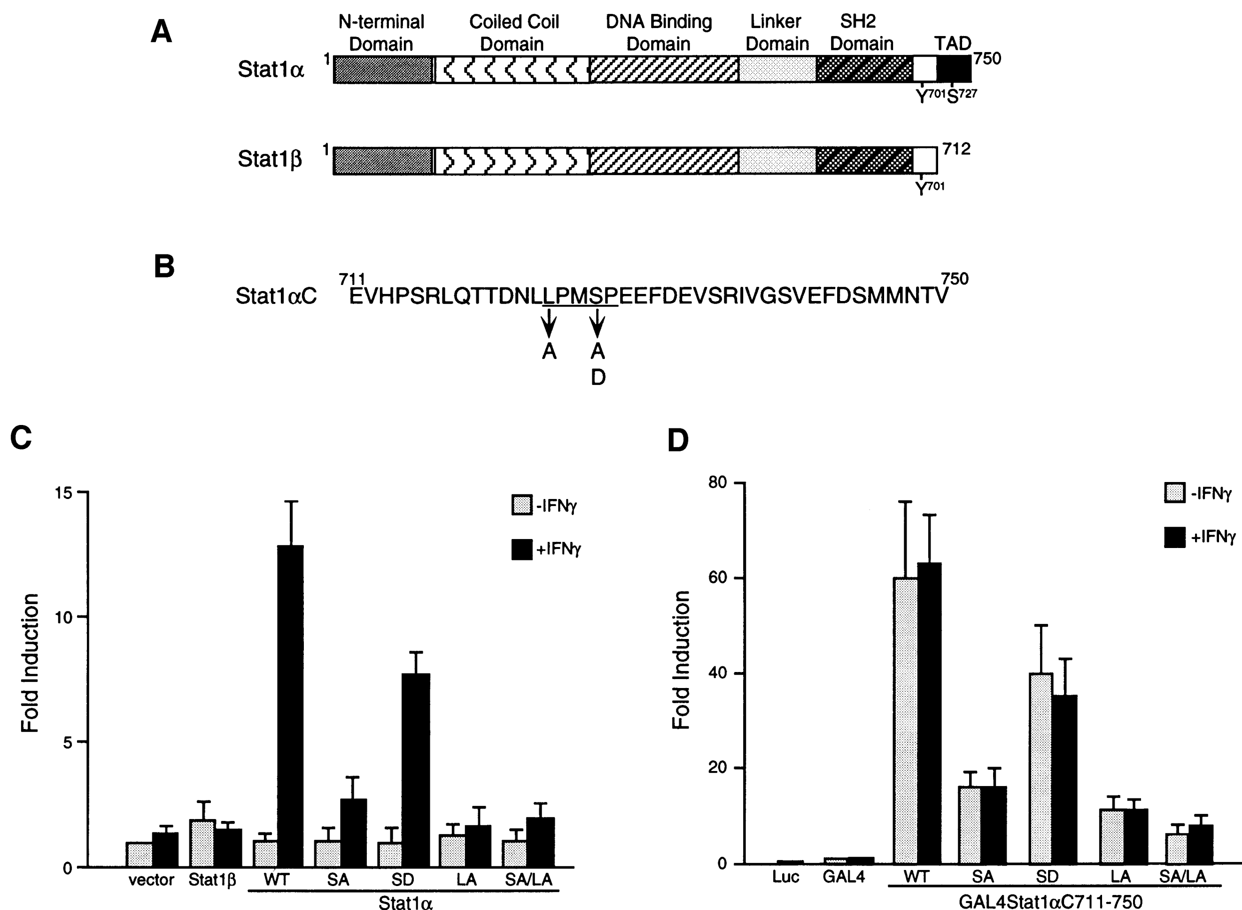

Fig. 1. The Stat1α C-terminus can act as an independent TAD. (**A**) Schematic view of two forms of Stat1 generated by alternative splicing: Stat1α and β. Domain structures are according to Chen *et al.* (1998). Y, Tyr701; S, Ser727; TAD, transcription activation domain. (**B**) Sequences of Stat1αC 711–750 with mutations of Ser727 and Leu724. Underlined sequence is the conserved XPMSP box (Wen *et al.*, 1995). (**C**) Effects of mutations of Ser727 and Leu724 on transcription activation by Stat1α. WT, wild-type; SA, Ser727 to alanine; SD, Ser727 to aspartic acid; LA, Leu724 to alanine. (**D**) The Stat1α C-terminus is an independent TAD. Transient transfections were performed in U3A cells with the 3xLy6E–GAS luciferase reporter (Wen *et al.*, 1995) for (C) and 5xGAL4DB luciferase reporter for (D). Twenty-four h after transfection, cells were left untreated or treated with IFN-γ for 6 h and harvested for luciferase assays. Results shown are the mean ± standard deviation of 3–6 experiments.

Stat1, Stat1 proteins containing point mutations of these residues (Figure 1A and B) were analyzed by transient transfection assays in U3A cells which lack endogenous Stat1 (Muller *et al.*, 1993). Equal amounts of these Stat1 mutant proteins were expressed (data not shown). Expression of Stat1β did not produce significant levels of reporter activity (Figure 1C). Stat1α induced an ~15-fold increase in reporter activity when the cells were treated with IFN-γ. Stat1α containing a mutation of S727A (Ser727 to alanine) induced a basal activity at ~25% of wild-type, as previously reported (Wen *et al.*, 1995). A point mutation of S727D (Ser727 to aspartic acid) increased the activity to a level equal to ~70% of the wild-type suggesting that phosphorylation of Ser727 serves to provide a negative charge on the molecule. Mutations of L724A and double mutations of S727A and L724A reduced the activity to that of Stat1β indicating the importance of Leu724 as well as Ser727 in transcriptional activation in response to IFN-γ.

DNA constructs encoding the DNA-binding domain of GAL4 fused to the wild-type residues 711–750 of Stat1α or to mutant versions of these residues (Figure 1B) were prepared to test the capacity of these residues to act independently as a TAD. GAL4 fusion proteins containing the wild-type C-terminal region of Stat1α (residues 711–750; Figure 1B) activated transcription >60-fold compared with the GAL4 DNA-binding domain alone, indicating that the C-terminal domain of Stat1α can function independently as a TAD (Figure 1D). Untreated cells gave similar reporter activity as treated cells probably due to the constitutive nuclear localization of the GAL4 fusion proteins (Nelson and Silver, 1989) and a constitutive level of phosphorylation on Ser727 when cells are grown in serum-containing medium. A point mutation of S727A caused a significant decrease in the activity of the fusion protein, indicating the importance of this serine residue for the optimal transcriptional activity of the TAD. Similar to the results seen with full-length Stat1α, mutations of S727D restored its activity to ~70% of the wild-type, while mutations of L724A or both S727A and L724A further reduced the transcriptional activity of the fusion protein. Similar results were obtained from transient transfection assays in NIH 3T3 cells (data not shown).

Together these results demonstrate that the residues Leu724 and Ser727 in the C-terminal region of Stat1α are essential for transcriptional activation by Stat1α, and

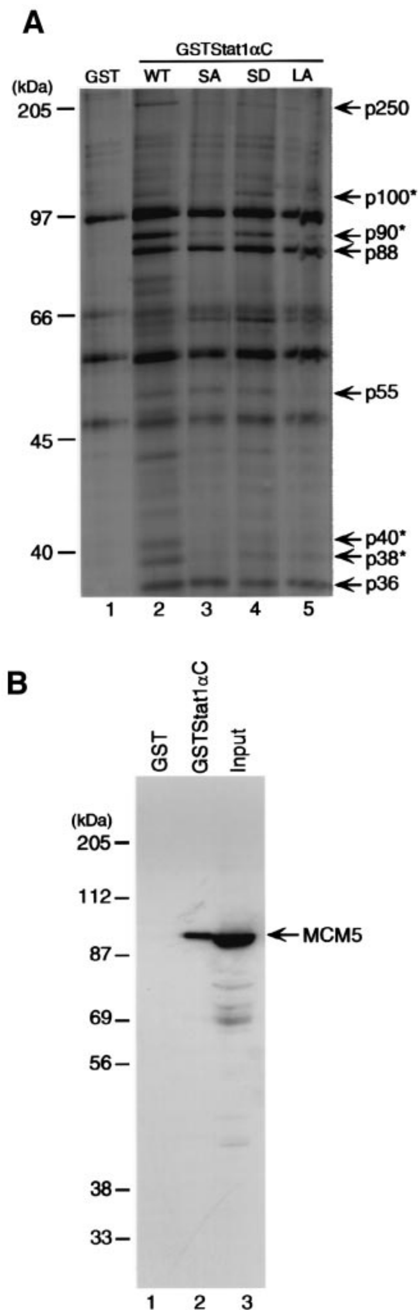

Fig. 2. MCM5 is a member of a group of nuclear proteins interacting with Stat1α TAD. (**A**) Interaction between a group of nuclear proteins with the Stat1α TAD. Nuclear proteins interacting with various GST fusion proteins were analyzed by SDS–PAGE and silver staining. GSTStat1αC, GST fusion proteins containing residues 711–750 of Stat1α; WT, wild-type; SA, Ser727 to alanine; SD, Ser727 to aspartic acid; LA, Leu724 to alanine. (**B**) MCM5 is one of the proteins interacting with the Stat1α TAD. Western blot analysis of the Stat1α TAD-interacting proteins was carried out with an MCM5-specific antibody (Paul *et al.*, 1996).

suggest specific interactions with phosphorylated Ser727 and Leu724 may be involved.

The Stat1α TAD interacts with a specific group of nuclear proteins

Glutathione *S*-transferase (GST) fusion proteins of the Stat1α TAD were used to detect interacting proteins with nuclear extracts from U3A cells (Figure 2A). A few proteins interacted with the GST protein alone (Figure 2A, lane 1) while many more proteins interacted with the GST fusion protein containing the wild-type Stat1α TAD, ranging in size from 38 to >200 kDa (Figure 2A, predominant bands are indicated by arrows and their estimated apparent molecular mass). Most significantly, point mutation of S727A greatly diminished the binding of at least four proteins (p38, p40, p90 and p100; Figure 2A, lane 3, indicated by *). Mutation of L724A likewise diminished the binding of most of the same proteins (Figure 2A, lane 5). The binding of at least p90 was restored by the S727D mutation (Figure 2A, lane 4). Thus there is a strong correlation between binding of some of these nuclear proteins and the transcriptional activation potential of the Stat1α TAD.

Western blot analyses with antibodies against many known proteins involved in transcription revealed p250 was CBP/P300 (data not shown), consistent with the results from earlier studies (Zhang *et al.*, 1996), but failed to identify any of the other bands in Figure 2A.

To determine the identity of the Stat1 TAD-interacting proteins, mass spectrometry was employed. After gel electrophoretic separation of the bound proteins, the p90 and p88 bands were recovered and subjected to in-gel trypsin digestion. The molecular masses of the tryptic peptides obtained from each protein band were measured by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. The resulting peptide mass map was used to identify proteins in the band of interest by searching the OWL protein sequence database with the program 'ProFound' (Fenyo *et al.*, 1996). This program determines the best match between an experimentally determined peptide mass map and a theoretically constructed map for each protein in the database (Henzel *et al.*, 1993; Fenyo *et al.*, 1996; Jensen *et al.*, 1996). The search yielded unambiguous identification of the p90 protein as the human MCM5 protein. Twenty-six peptides were found to match calculated molecular masses of theoretical tryptic peptides from human MCM5 with mass accuracy better than ± 0.1 dalton (accounting for 49% of the protein sequence; Fenyo *et al.*, 1996). While a large number of discrete peptides were also obtained in the digest of p88, no match could be found in the database as of June 1, 1998.

To confirm that p90 was in fact MCM5, Western blot analysis of the proteins interacting with GSTStat1αCWT was performed using an MCM5-specific antibody (Paul *et al.*, 1996). A single band at ~90 kDa was detected in the proteins bound by Stat1α TAD (Figure 2B, lane 2) and in the total nuclear protein extract (Figure 2B, lane 3) while there was no detectable signal in the GST-alone lane (Figure 2B, lane 1), demonstrating that MCM5 is a member of a group of proteins interacting specifically with the Stat1α TAD. MCM5 has been shown to interact strongly with another member of the MCM family, MCM3 (Kimura *et al.*, 1995; Schulte *et al.*, 1995). Western blot analysis with an MCM3-specific antibody (Schulte *et al.*, 1995) showed that p100 is MCM3 (data not shown). However, MCM3 is present at a much lower level than MCM5 in this group of Stat1-bound proteins (Figure 2A and Western blot data not shown) and no other MCM members were detected (data not shown). These results suggest that the MCM5 protein in the Stat1TAD-interacting

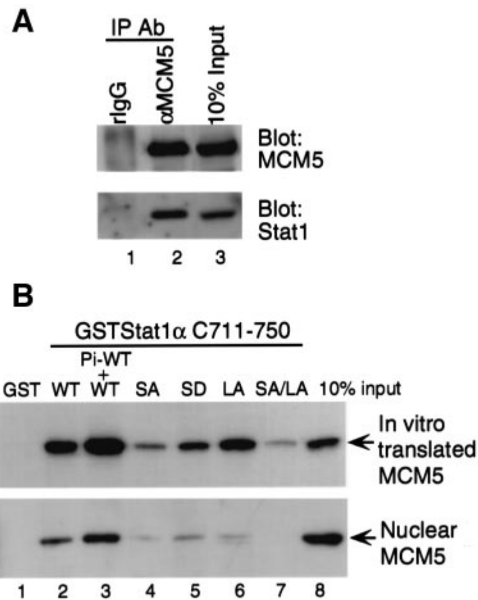

Fig. 3. Stat1α interacts with MCM5 *in vivo* and *in vitro*. (**A**) *In vivo* interaction between Stat1α and MCM5. Nuclear extracts from IFN-γ-treated 2fTGH cells were immunoprecipitated with the antibodies indicated and the resulting immune complexes were analyzed by Western blotting. IPAb, immunoprecipitating antibodies; rIgG, rabbit immunoglobulin. (**B**) Ser727- and Leu724-dependent interaction between Stat1α TAD and MCM5 *in vitro*. MCM5 bound to various GST fusion proteins were analyzed by SDS–PAGE and visualized by autoradiography of ^{35}S-labeled MCM5 proteins translated *in vitro* (top panel), or by Western blotting with an MCM5 antibody of MCM5 from nuclear extracts of IFN-γ-treated 2fTGH cells (bottom panel). WT, wild-type; Pi-WT, wild-type with Ser727 phosphorylated *in vitro*; SA, Ser727 to alanine; SD, Ser727 to aspartic acid; LA, Leu724 to alanine.

complex is not part of the usual MCM complexes involved in DNA replication.

Interactions between Stat1α and MCM5 in vivo and in vitro

Co-immunoprecipitation experiments were performed using nuclear extracts form 2fTGH cells, the parental cell line for U3A cells and containing both wild-type Stat1α and MCM5 proteins. Only nuclear extracts from IFN-γ-treated cells were used as there is very little Stat1 protein in the nucleus of untreated cells. A significant amount of the total nuclear MCM5 was precipitated by the MCM5 antibody (Figure 3A, top panel, lane 2) while no detectable MCM5 was present in the precipitate with a control antiserum (Figure 3A, top panel, lane 1). While the control antiserum did not precipitate either Stat1 or MCM5 (Figure 3A, lane 1), Stat1α protein was present in the immune complex precipitated by the MCM5 antibody (Figure 3A, bottom panel, lane 2) indicating an *in vivo* interaction between Stat1α and MCM5 in the nucleus.

In vitro binding analyses were carried out using GST fusion proteins containing the Stat1 TAD with full-length MCM5 protein translated *in vitro* (Figure 3B, top panel) or with nuclear extracts of IFN-γ-treated 2fTGH cells (Figure 3B, bottom panel). GST alone does not interact with MCM5 (Figure 3B, lane 1), but a significant amount of the *in vitro* translated MCM5 and the nuclear MCM5 interacted with the GSTStat1αCWT (Figure 3B, lane 2), indicating a direct interaction between Stat1TAD and MCM5. The GSTStat1αCWT protein was also phosphorylated on Ser727 *in vitro* using a cytoplasmic fraction containing a serine kinase activity that correctly phosphorylates Ser727 (Zhu *et al.*, 1997). The mixture containing serine-phosphorylated GSTStat1αCWT interacted more intensely with both types of MCM5 (Figure 3B, lane 3). Both types of MCM5 interacted much less significantly with the S727A mutant (Figure 3B, lane 4), or with the S727A/L724A double mutant protein (Figure 3B, lane 7), while a higher amount of MCM5 interacted with the S727D mutant (Figure 3B, lane 5), all of which were consistent with the results of Figure 2A. However, the *in vitro* translated MCM5 interacted with the L724A mutant almost as strongly as the wild-type (Figure 3B, top panel, lane 6) while the nuclear MCM5 did not (Figure 3B, bottom panel, lane 6). This possibly reflects post-translational modifications, e.g. phosphorylation, of the nuclear MCM5 (Musahl *et al.*, 1995) which are not present in the *in vitro* translated protein. GST fusion proteins of the C-terminus of Stat3 and Stat4 did not interact with either type of MCM5 by this assay (data not shown).

Specific regions in MCM5 interact with the Stat1α TAD

As a further test of the specificity of MCM5–Stat1 TAD interaction, the location of a specific interactive region or regions of MCM5 was sought. MCM5 was subcloned into a vector with a T7 promoter at the 5′ end of the coding region. Unique restriction sites in the coding region of MCM5 were utilized to linearize the plasmid to generate different lengths of the protein which could be labeled by *in vitro* translation (Figure 4A). The resulting labeled protein fragments were then tested for their ability to bind to the GSTStat1αCWT fusion protein. The full-length MCM5 interacted with GSTStat1αCWT strongly (Figure 4B, lane 20) while deletion of the last 10 residues in the C-terminal region of MCM5 greatly reduced the binding affinity (Figure 4B, lane 17). However, a low level of binding persisted in the N-terminal half of the MCM5 molecule to residues 1–401 (Figure 4B, lanes 8, 11 and 14). Fragments containing residues 1–135 and 1–254 failed to interact with GSTStat1αCWT (Figure 4B, lanes 2 and 5). Further analyses showed that fragments containing residues 201–400 or 251–401 of MCM5 were sufficient to interact with GSTStat1αCWT (Figure 4B, lanes 23 and 26) while a fragment containing residues 651–734 could not bind GSTStat1αCWT (data not shown). Together, these results indicate the region between residues 251 and 401 of MCM5 is sufficient for binding Stat1α and that the C-terminus of MCM5 is also important for optimal interaction.

MCM5 enhances transcriptional activation by Stat1α

To examine the possibility that MCM5 is involved in transcriptional activation by Stat1α, MCM5 was transiently over-expressed in U-2 OS cells (~50% of the cells become transfected, data not shown), achieving an overall 2- to 3-fold increase in the total level of MCM5 in the culture [Figure 5A, bottom panel; only IFN-γ-treated samples are shown as MCM5 level is not affected by IFN-γ treatment (data not shown)]. IFN-γ treatment induced a

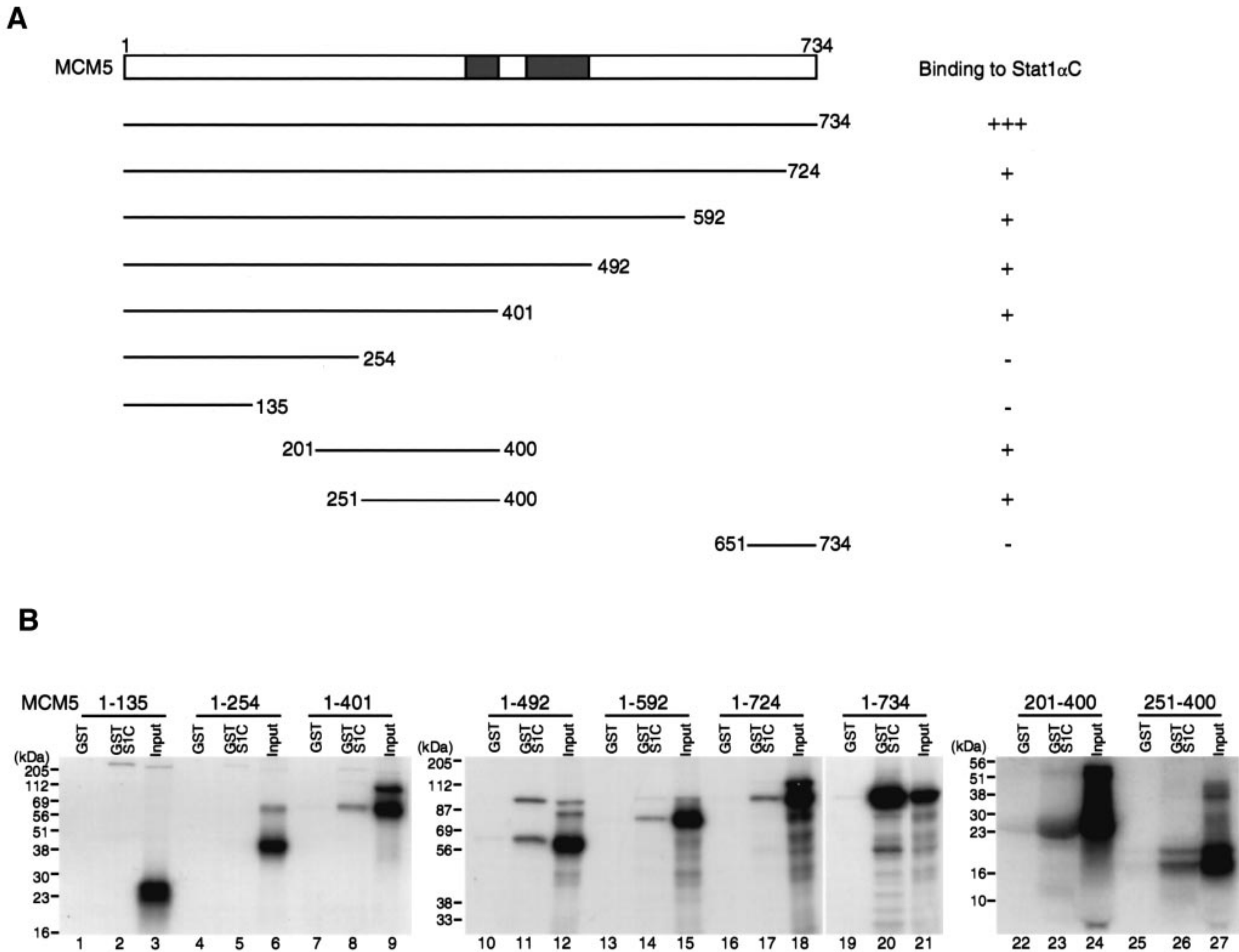

Fig. 4. Regions of MCM5 that interact with Stat1α TAD. (**A**) Schematic view of MCM5 and a summary of the interaction between MCM5 and GSTStat1αC. The various DNA fragments used for *in vitro* translation are as indicated and the binding results summarized on the right. Shaded boxes indicate DNA-dependent ATPase domains. (**B**) Interactions of Stat1α TAD with various regions of MCM5. GST, glutathione-*S*-transferase; GSTS1C, GST fusion protein of Stat1α 711–750. The various translated proteins were incubated with Sepharose beads bound with GST or GSTS1C. The proteins bound on beads were separated by SDS–PAGE and visualized by autoradiography.

reporter activity ~20-fold that of untreated cells. While over-expression of MCM5 did not affect reporter activities from untreated cells significantly, IFN-γ-induced activity was increased with increasing addition of MCM5 (Figure 5A, top panel), suggesting MCM5 is directly involved in transcription activation by Stat1α in response to IFN-γ.

To test for the requirement of Stat1 TAD in the MCM5-dependent transcriptional enhancement, stable cell lines of the Stat1-deficient U3A cells complemented with Stat1β, Stat1α or Stat1α containing the S727A mutation (Stat1αSA) (Wen *et al*., 1995) were utilized (Figure 5B). The increase of total MCM5 in these cells enhanced the IFN-γ induced reporter activity ~4-fold in cells containing wild-type Stat1α, but not in cells containing Stat1β. In Stat1αSA cells, IFN-γ treatment induced a transcriptional activity ~20% that of the wild-type Stat1α (Wen *et al*., 1995) (Figure 1C). However, this activity was not enhanced by the over-expression of exogenous MCM5 (Figure 5B, top panel). Reporter activities in untreated cells were not affected (data not shown). Western blot analyses showed similar increase in levels of MCM5 in all three cell types (Figure 5B, bottom panel). Thus a Ser727-dependent interaction between Stat1α and MCM5 is required *in vivo* for the enhancement of transcriptional activity of Stat1α by MCM5.

Stat1α transcriptional activity correlates with the change in level of endogenous nuclear MCM5 during the cell cycle

It has been reported that there is a change in the level of nuclear localized MCM proteins during cell-cycle progression, i.e. the level of nuclear localized MCMs increases during G_1 and decreases during S phase (Kimura *et al*., 1995; Todorov *et al*., 1995; Fujita *et al*., 1996; Krude *et al*., 1996). We sought to utilize this change in the level of endogenous MCM5 to investigate further the effect of different levels of nuclear MCM5 on the transcriptional activity of Stat1α.

The 3×Ly6E luciferase reporter construct was stably integrated in 2fTGH cells for analyzing IFN-γ-induced transcription activity. This provides a chromosomal reporter gene which is dependent solely on Stat1 activity. Cells were synchronized at G_2/M by 16 h of treatment with nocodazole and released from the block for different lengths of time. For sufficient accumulation of reporter

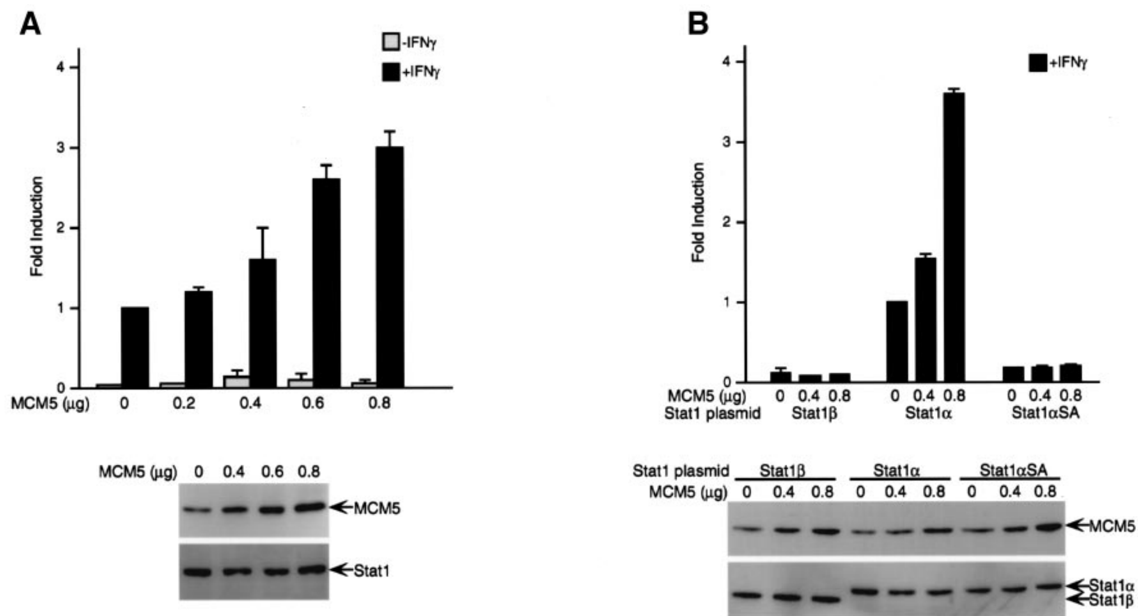

Fig. 5. MCM5 enhances IFN-γ-induced transcriptional activation by Stat1α. (**A**) Over-expression of MCM5 increases IFN-γ-induced reporter activity by Stat1α. Transient transfections of an MCM5 expression plasmid were performed in U-2 OS cells together with the 3×Ly6E luciferase reporter. Twenty-four h after transfection, cells were left untreated or treated with IFN-γ for 6 h and harvested for luciferase assays. Results shown are the mean ± standard deviation of 3–6 experiments. Western blot analyses were carried out with 10 μl of cell lysate from IFN-γ-treated samples as indicated. (**B**) Enhancement of transcription activation of Stat1α by MCM5 depends on Ser727. U3A cells complemented with Stat1β, Stat1α and Stat1α S727A (Wen *et al*., 1995) were used for transient transfection assays as in Figure 5A. Only results from IFN-γ treated cells are shown.

luciferase proteins, cells were treated with IFN-γ for 3 h at different time periods during the release or left untreated and then harvested for luciferase assays, Western blotting and Northern blotting. In addition, the population of cells at different stages of the cell cycle at each time point for this experiment were determined by propodium iodide (PI) staining and fluorescence-activated cell sorting (FACS) analyses (data not shown).

Asynchronously growing cells (Figure 6A, normal) gave a 4-fold induction of reporter activity when treated with IFN-γ for 3 h, while cells blocked at G_2/M (Figure 6A, t_0) produced <2-fold induction of reporter activity. Within 3 h of release from nocodazole block, the majority of the cells entered the G_1 phase (FACS data not shown) and the reporter activity was induced ~2-fold by the IFN-γ treatment between the 0 and 3 h point. Cells released from nocodazole block for 6 h were mostly in G_1 or at the G_1/S phase boundary (data not shown), and when treated with IFN-γ from 3–6 h after release, gave a 15-fold induction of reporter activity. Cells released from nocodazole block for 9 h were mostly in S phase (FACS data not shown) and when treated with IFN-γ from 6–9 h after release, gave an 8-fold induction of reporter activity. After releasing from the nocodazole block for 24 h, the cells had again become asynchronous (data not shown) and gave a similar induction of reporter activity to that of normally growing cells (Figure 6A, 24 h).

Western blot analyses were performed with nuclear extracts from cells collected at different time points during the release from G_2/M block. Corresponding to 0 h in Figure 6A, cells at G_2/M block contained low levels of nuclear MCM5 (Figure 6B, lane 3) compared with asynchronously growing cells (Figure 6B, lanes 1 and 2). Cells within 3 h of release (Figure 6B, lanes 4 and 5, corresponding to the 3 h point sample in Figure 6A) also contained low levels of nuclear MCM5. The level of nuclear MCM5 was highest in cells released for 3–5 h (Figure 6B, lanes 6–8, corresponding to the 6 h point sample in Figure 6A). After releasing for more than 6 h, the level of nuclear MCM5 decreased (Figure 6B, lanes 9–12, corresponding to the 9 h point sample in Figure 6A). Cells released for 24 h (Figure 6B, lane 13) contained similar levels of nuclear MCM5 to that of the asynchronously growing cells (Figure 6B, lanes 1 and 2). Except for the G_2/M blocked cells and cells within the first hour of release which contained a lower level of tyrosine phosphorylated Stat1α, cells at different time points were capable of inducing tyrosine phosphorylation on Stat1α in response to a 15-min treatment with IFN-γ to similar levels (Figure 6B, bottom panel). Cells not treated with IFN-γ did not show significant difference in reporter activity (Figure 6A) and contained similar levels of nuclear localized MCM5 as treated cells at each time point (data not shown).

Northern blot analyses of the IFN-γ-responsive IRF-1 gene and the constitutive GAPDH gene were performed with RNA samples collected at the same time points as in Figure 6A. The accumulation of IRF-1 mRNA, which is the result of transcriptional regulation in response to IFN-γ treatment, showed a similar expression pattern to that of the luciferase reporter activity in Figure 6A, while expression of GAPDH was not affected (Figure 6C). Taken together, these results strongly confirm the correlation between the increase in the level of nuclear localized MCM5 protein and the transcription activation potential of Stat1α in response to IFN-γ.

Discussion

Earlier studies have led to the hypothesis that the C-terminal region of Stat1α is the TAD (Muller *et al*.,

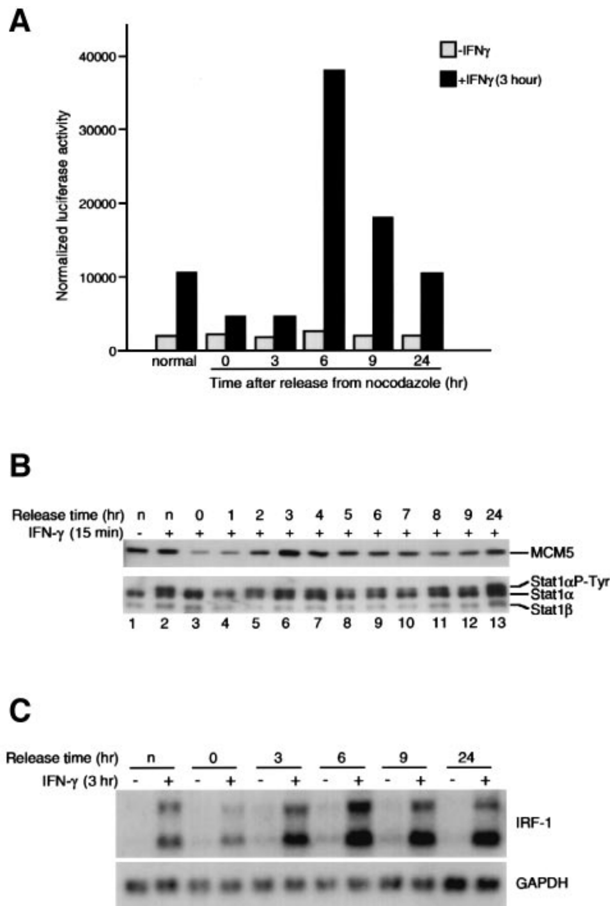

Fig. 6. Stat1α transcriptional activity correlates with the change in level of endogenous nuclear MCM5 during the cell cycle. (**A**) The IFN-γ-induced transcriptional activity by Stat1α changes during the cell cycle. Cells (2fTGH) containing a stably-integrated 3×Ly6E luciferase reporter were synchronized at G_2/M by nocodazole and released for different lengths of time as indicated. Cells were left untreated or treated with IFN-γ for 3 h prior to harvesting for luciferase assays. Similar results were obtained with four independent lines and one representative result is shown. Normal, asynchronously growing cells. (**B**) Western blot analyses of levels of nuclear localized MCM5 and Stat1. Nuclear extracts were prepared from 2fTGH cells at different time points as indicated and analyzed by Western blotting. n, asynchronously growing cells; P-Tyr, Stat1α with a phosphorylated Tyr701. (**C**) Northern blot analyses of IRF-1 and GAPDH transcripts. RNA samples were prepared from 2fTGH cells collected at time points indicated and analyzed by Northern blotting with probes of human IRF-1 and GAPDH cDNA.

1993; Wen *et al*., 1995). In this report, we demonstrate that this domain can be isolated and act as an independent TAD when fused to the GAL4 DNA-binding domain. Mutational analyses showed that in addition to Ser727, Leu724 is also important for optimal transcription activity of Stat1α. The effect of mutation of Ser727 to aspartic acid suggests that phosphorylation of this serine probably provides the Stat1α molecule with a negative charge for optimal transcription activation. A specific group of nuclear proteins are shown to interact with this TAD, some in a Ser727- and Leu724-dependent manner. The exact correlation between the binding affinity to these proteins and the transcription activation potential of the Stat1α C-terminus suggests strongly that these interactions have functional importance in transcription activation by Stat1. Furthermore, the phosphorylation of Ser727 increases the binding affinity for these nuclear proteins, providing a mechanistic explanation for the earlier findings that phosphoserine-727 is required for maximal transcription activation by Stat1α (Wen *et al*., 1995). A surprising result from current experiments is the strong interaction of the Stat1α TAD with MCM5, a protein shown previously to be involved in DNA replication. The most convincing evidence of the importance of this interaction for transcriptional activation was the correlation of IFN-γ-inducible transcription with the amount of nuclear MCM5.

Previous functional studies have shown the MCMs to form complexes of various constitutions (Fujita *et al*., 1997; Richter and Knippers, 1997; Holthoff *et al*., 1998), and to associate with initiation and elongation complexes during DNA replication (Aparicio *et al*., 1997; Tanaka *et al*., 1997). The MCMs have common structural features such as DNA-dependent ATPase domains characteristic of DNA helicases (Koonin, 1993). They exist in the cell at a level ~50- to 500-fold higher than the number of DNA replication origins in yeast (Lei *et al*., 1996; Donovan *et al*., 1997) suggesting that their putative helicase activity could be involved in both replication and in DNA repair or recombination (Newlon, 1997). They have been shown to change the structure of chromatin (Ishimi *et al*., 1996; Holthoff *et al*., 1998) and/or unwind DNA with their potential helicase activity (Ishimi, 1997). These functions could be important for the initiation of both DNA replication and RNA transcription. The results presented here suggest that Stat1α, using the conserved Ser727 and Leu724 residues, recruits MCM5 for optimal transcriptional activation in response to IFN-γ.

A final point concerns a potential direct role of the interaction between Stat1α and MCM5 in cell-cycle regulation, differing from the demonstrated role in transcription. IFN-γ treatment slows cell growth, specifically the entrance of cells into S phase. This slowing of the cell cycle requires the presence of wild-type Stat1α in the cell (Bromberg *et al*., 1996). It may be that upon treatment with IFN-γ, the significant increase in the amount of nuclear Stat1α interacting with MCM5 could redistribute MCM5 from DNA replication to transcription, hence causing the block of entrance into S phase. The interaction between Stat1α and MCM5 thus could have a dual role in two diverse cellular processes, both affected in response to treatments of IFN-γ.

Materials and methods

Cell culture and antibodies

Cell-types 2fTGH and U3A (provided by George Stark, Cleveland Clinic Foundation Research Institute, OH, and Ian Kerr, Imperial Cancer Research Foundation, UK) were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% cosmic calf serum (Hyclone Laboratories Incorporated). U-2 OS human osteosarcoma cells (purchased from ATCC) were maintained in DMEM supplemented with 10% fetal bovine serum (Hyclone Laboratories Incorporated). U3A cells with stably transfected expression plasmids containing Stat1α, Stat1β, Stat1αSA were maintained in G418 at 0.5 mg/ml (Gibco-BRL). The MCM5-specific polyclonal antibody was made against bacterially expressed MCM5 protein and affinity-purified (Paul *et al*., 1996). An antibody against the N-terminus of Stat1 was purchased from Transduction Laboratory. Control antibody rabbit IgG was purchased from Santa Cruz. Recombinant human IFN-γ was a gift from Amgen. Cells were treated with IFN-γ at 5 ng/ml for lengths of time as indicated in each experiment. For cell-cycle blocking experiments, cells at 80% confluency were treated with nocodazole (Sigma) at 100 ng/ml for 16 h. Mitotic-

arrested cells were pipetted off the plates, washed with phosphate-buffered saline and plated in normal media for further analyses.

GST-fusion protein interaction assays, co-immunoprecipitation and Western blot analysis

GST fusion proteins were purified from bacteria using glutathione–Sepharose beads as instructed by the manufacturer (Pharmacia). Nuclear extracts were prepared as previously described (Zhang *et al*., 1996). *In vitro* translation reactions were carried out using the TNT T7 system as instructed by the manufacturer (Promega). For binding assays in Figure 2, various GST fusion proteins were bound on a 20 μl Sepharose column at 3 μg protein/μl beads. Nuclear extracts from U3 cells were dialyzed in BC100 buffer (50 mM Tris–HCl pH 8, 0.5 mM EDTA, 100 mM NaCl and 20% glycerol) and applied to the GST fusion protein columns. The columns were then washed with BC100 containing 0.1% nonident P40 and nuclear proteins bound on the columns were eluted by high-salt buffer BC1000 (1 M NaCl). Proteins in the various eluates were separated by SDS–PAGE and visualized by silver staining using the Rapid-Silver-Stain kit according to the manufacturer (ICN). Binding assays in Figures 3 and 4 were performed as previously described (Zhang *et al*., 1996). For co-immunoprecipitation experiments, nuclear extracts from IFN-γ-treated (30 min) 2fTGH cells were dialyzed with BC100 and incubated overnight with 5 μg of antibodies in 50 mM Tris pH 7.5, 150 mM NaCl, 0.1% Triton-X, 0.1% SDS and 0.1% Brij-96. Immune complexes were brought down with protein A agarose beads, washed with the same buffer and separated by SDS–PAGE. Western blot analyses were carried out using chemiluminescence as instructed by the manufacturer (Dupont/New England Nuclear). Serine phosphorylation reactions of GSTStat1αCWT were performed as previously described (Zhu *et al*., 1997) with 15 μg of purified GST fusion protein and 5 μl of the serine kinase-containing fraction. The phosphorylated GST fusion proteins were then re-purified with glutathione–Sepharose beads (Promega). The specificity of phosphorylation on Ser727 was indicated by a much greater ^{32}P-incorporation into GSTStat1αC wild-type protein than into the GSTStat1αC S727A mutant protein (data not shown). (The extent of serine phosphorylation in the wild-type fusion protein is not known but may well not be complete.)

Mass spectrometry

The separated proteins in SDS–PAGE gel were visualized by copper-staining (Bio-Rad Laboratories, Hercules, CA). The bands of interest were excised, subjected to in-gel digestion with trypsin and the resulting proteolytic mixtures extracted as described previously (Qin *et al*., 1997). The molecular masses of the peptide mixtures were accurately determined by MALDI-TOF mass spectrometry using an instrument which incorporates delayed ion extraction and ion reflection (Voyager-DE STR, Perseptive Biosystems, Inc., Framingham, MA). The complete list of accurately measured masses of the tryptic peptides was used to search for protein candidates in the OWL protein sequence database with the program 'ProFound' (http://prowl/cgi-bin/ProFound; Fenyo *et al*., 1996).

Plasmid constructions

GST fusion constructs containing the C-terminal region of Stat1α were constructed from PCR products of residues 711–750 of Stat1α using *Bam*H1 and *Xho*I sites in the pGEX-5x-1 vector (Pharmacia). Mutations of Ser727 to aspartic acid, glutamic acid and double mutations of Ser727/Leu724 were generated by PCR with oligonucleotides containing the appropriate mutations. The same fragments were also cloned into the pSG424 vector for GAL4 fusion proteins at the *Sac*I site (Sadowski and Ptashne, 1989). Construction of expression vectors Rc/CMV (Invitrogen) containing Stat1α, Stat1β and Stat1αSA was as previously described (Wen *et al*., 1995). Expression plasmid Rc/CMVStat1αLA was a gift from Z.Wen (UCSF, CA). Rc/CMVStat1αSD, SA/LA were prepared by replacing the *Xba*I/*Apa*I fragment of Rc/CMVStat1αWT with PCR fragments of Stat1α711–750 containing the appropriate mutations. The 3xLy6E luciferase reporter was constructed as previously described (Wen *et al*., 1995). The GAL4 luciferase reporter was a gift from W.Gu (The Rockefeller University, NY). The full-length cDNA of MCM5 was cloned into *Bam*HI and *Hin*dIII sites of pRSETB vector (Invitrogen). The MCM5 expression plasmid was constructed with a *Not*I and *Apa*I (partial digestion) fragment of full-length MCM5 cDNA into the Rc/CMV mammalian expression vector (Invitrogen).

RNA preparation and Northern blot

Total RNA was prepared using the TRIzol single-step RNA isolation method as instructed by the manufacturer (Gibco) and Northern blot analyses were carried out as described previously (Wen *et al*., 1995).

Transfection experiments

Transient transfections were performed in duplicate on 24-well plates with 5×10^4 cells per well or 6-well plates with 1.5×10^5 cells per well using the calcium phosphate method or LipofectAMINE Plus method as instructed by the manufacturer (Gibco-BRL). Twenty-four h after transfection, cells were treated with IFN-γ for 6 h or left untreated. Luciferase assays were performed using the dual-luciferase reporter system according to the manufacturer (Promega). Except for Figure 6A, all results shown are luciferase activities normalized against an internal control luciferase reporter of Renilla luciferase (Promega). Results in Figure 6A were normalized with total protein concentration determined by Bio-Rad protein assays according to the manufacturer.

Acknowledgements

We thank George Stark and Ian Kerr for the U3A and 2fTGH cell lines, Wei Gu for the GAL4 DNA-binding domain fusion vector and GAL4 luciferase reporter, Zilong Wen for Rc/CMVStat1αLA plasmid, Jackie Bromberg for IRF-1 and GAPDH plasmids and Lois Cousseau for preparing the manuscript. This work was supported by NIH grants AI34420 and AI32489 to J.E.D.; NIH grant RR00862 and NSF grant 9630936 to B.T.C. J.J.Z. was supported by a Postdoctoral Fellowship from the Aaron Diamond Foundation.

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Received August 24, 1998; revised October 1, 1998; accepted October 2, 1998