Oncogene (2000) 19, 5041–5053

www.nature.com/onc

Interferon-β induces S phase slowing via up-regulated expression of PML in squamous carcinoma cells

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Type I Interferon (IFN) and all-trans retinoic acid (RA) inhibit cell proliferation of squamous carcinoma cell lines (SCC). Examinations of growth-affected cell populations show that SCC lines ME-180 and SiHa treated with IFN-β undergo a specific slower progression through the S phase that seems to trigger cellular death. In combination treatment RA potentiates IFN-β effect in SCC ME-180 but not in SiHa cell line, partially resistant to RA antiproliferative action. RA added as single agent affects cell proliferation differently by inducing a slight G_1 accumulation. The IFN-β-induced S phase lengthening parallels the increased expression of PML, a nuclear phosphoprotein specifically up-regulated at transcriptional level by IFN, whose overexpression induces cell growth inhibition and tumor suppression. We report that PML up-regulation may account for the alteration of cell cycle progression induced by IFN-β in SCC by infecting cells with PML-PINCO recombinant retrovirus carrying the PML-3 cDNA under the control of the 5′ LTR. In fact PML overexpression reproduces the IFN-β-induced S phase lengthening. These findings provide important insight into the mechanism of tumor suppressing function of PML and could allow PML to be included in the pathways responsible for IFN-induced cell growth suppression. *Oncogene* (2000) **19,** 5041–5053.

Keywords: Interferon; all-trans retinoic acid; PML; cell cycle; squamous carcinoma cells

Introduction

Type I Interferon (IFN) and all-trans retinoic acid (RA) inhibit cell proliferation of squamous carcinoma cell (SCC) lines and induce apoptotic cell death (Lancillotti *et al*., 1995; Kalemkerian and Ramnath, 1996; Romeo and Affabris, 1996; Lindner *et al*., 1997; Lippman *et al*., 1997; Giandomenico *et al*., 1997, 1998; Chelbi-Alix and Pelicano, 1999; Percario *et al*., 1999). The aim of this work was the understanding of mechanisms underlying IFN treatment of neoplasms also in combination with retinoic acid.

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Received 25 May 2000; revised 28 July 2000; accepted 22 August 2000

The control of cell proliferation is increasingly being seen as a result of the signaling mechanisms leading to growth stimulation (Wolthuis and Bos, 1999), growth inhibition or death induced by tumour suppressor genes (Sharpless and DePinho, 1999).

Various antimitogenic factors including TGF-β, cAMP, rapamycin (Matsuoka *et al*., 1998) as well as IFN and RA (Iwase *et al*., 1997; Subramaniam *et al*., 1998; Lee *et al*., 1998; Qin *et al*., 1997; Kuniyasu *et al*., 1997) induce cell growth arrest by affecting cell cycle progression. The cell cycle progression control by various extracellular growth modifiers involves common mediators of various biological processes including DNA damages and apoptosis (Dulic *et al*., 1994), cell differentiation (Liu *et al*., 1996b), cell to cell contact inhibition (Polyak *et al*., 1994), cell senescence (Hara *et al*., 1996). Oncogenic and anti-oncogenic signals (Lloyd *et al*., 1997; Dyson and Balmain, 1999) converge to cyclin/CDK complexes, CDK inhibitors, E2F transcription factor family and retinoblastoma (Rb) family (Macleod, 1999).

PML is a ubiquitously expressed, matrix-associated nuclear phosphoprotein whose overexpression induces cell growth inhibition and tumor suppression (Mu *et al*., 1994; Chang *et al*., 1995; Koken *et al*., 1995; Fagioli *et al*., 1998). The mechanisms by which PML suppresses tumorigenesis and cell proliferation are poorly understood. PML is typically localized within discrete speckled nuclear structures called nuclear bodies (NBs) or PODs (for PML oncogenic domains) (Lamond and Earnshaw, 1998; Hodges *et al*., 1998) and is specifically up-regulated at transcriptional level by IFNs (Stadler *et al*., 1995; Lavau *et al*., 1995; Nason-Burchenal *et al*., 1996). The functions of NBs are still unknown (Seeler and Dejean, 1999), but they seem to be involved in transcriptional regulation. Nascent RNA polymerase II transcripts have been shown to localize within the NBs (LaMorte *et al*., 1998). Moreover, PML appears to cooperate in transcriptional activation mediated by AP-1 (Vallian *et al*., 1998), retinoid receptors (Wang *et al*., 1998a) and glucocorticoid receptor (Doucas *et al*., 1999). Analysis of the 5′ regulatory sequences of PML revealed both a functional IFN-α/β-stimulated response element, ISRE, and an IFN-γ activation site, GAS (Stadler *et al*., 1995), demonstrating that PML is a primary target gene of IFNs. NB-associated proteins i.e.

PML (Chelbi-Alix *et al*., 1995, 1998; Lavau *et al*., 1995; Stadler *et al*., 1995), Sp100 and Sp140, autoantigens of primary biliary cirrhosis (Szostecki *et al*., 1990; Guldner *et al*., 1992; Bloch *et al*., 1999) as well as ISG20 (Gongora *et al*., 1997) are all IFN induced, suggesting a critical role for this nuclear structure in the IFN response. In addition, PML is responsible for the proper localization of all NBs-associated proteins since they appear dispersed in $PML^{-/-}$ cells (Ishov *et al*., 1999). Thus, an important point is to find which of IFN biological effects could be mediated by PML. Investigations of proteins that interact with PML within NBs showed that PML forms complexes with the unphosphorylated form of pRB (Alcalay *et al*., 1998). In PML knockout mice and cells it has been shown that PML antagonizes the initiation, promotion and progression of tumors of various histological origins (Wang *et al*., 1998a). PML may exert its growth suppressing effect by modulating cell cycle distribution (Le *et al*., 1998) and it appears essential for induction of apoptosis by multiple stimuli including IFN (Wang *et al*., 1998b; Quignon *et al*., 1998).

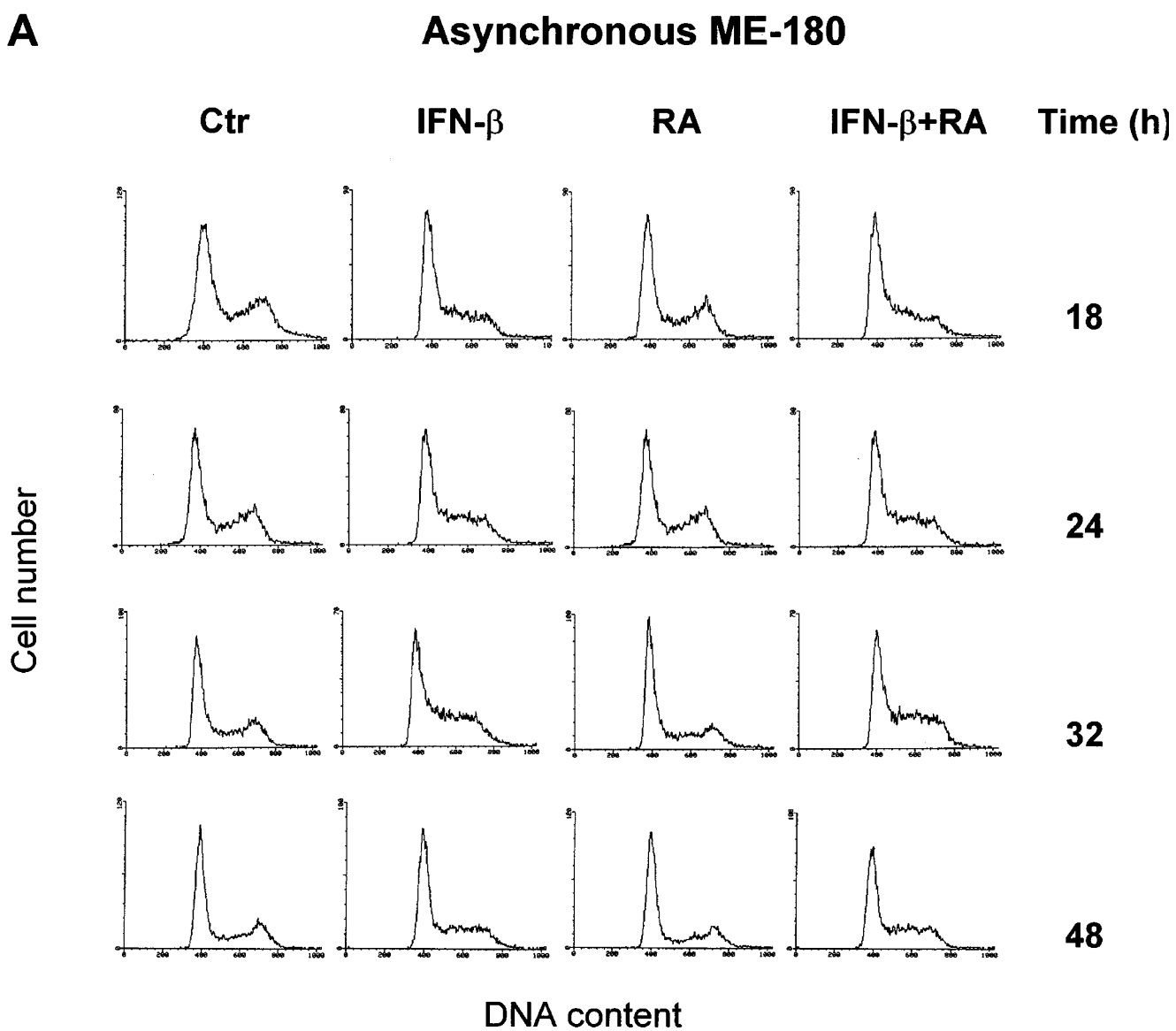
We show that IFN-β and RA exert their anticellular effects in SCC by differently affecting progression of cells through the cell cycle. In particular, RA induces a slight accumulation in G_1-phase, whereas IFN-β dramatically slows progression of cells through the S phase. IFN-β-induced S phase alteration is associated with PML protein upregulation and marked cell growth inhibition.

Therefore, we provide a molecular basis underlying this phenomenon by demonstrating that PML overexpression reproduces the S phase slowing observed by IFN-β treatment, including PML in the pathways of IFN-induced cell growth suppression.

Results

IFN-β induces a lengthening of cell cycle S phase

To answer the question if IFN-β and RA antiproliferative effects involve some alteration in cell cycle progression, we treated ME-180 and SiHa squamous carcinoma cells with 50 IU/ml IFN-β,



10^{-6} M RA and their combination for several time points. Total populations were then assayed for DNA content by flow cytometry and for proliferation rates.

As represented in Figure 1a,b, both cell lines showed a significant accumulation in S phase when treated with IFN-β. Particularly, the increase of S phase cell population was evident after 18 h in ME-180 and

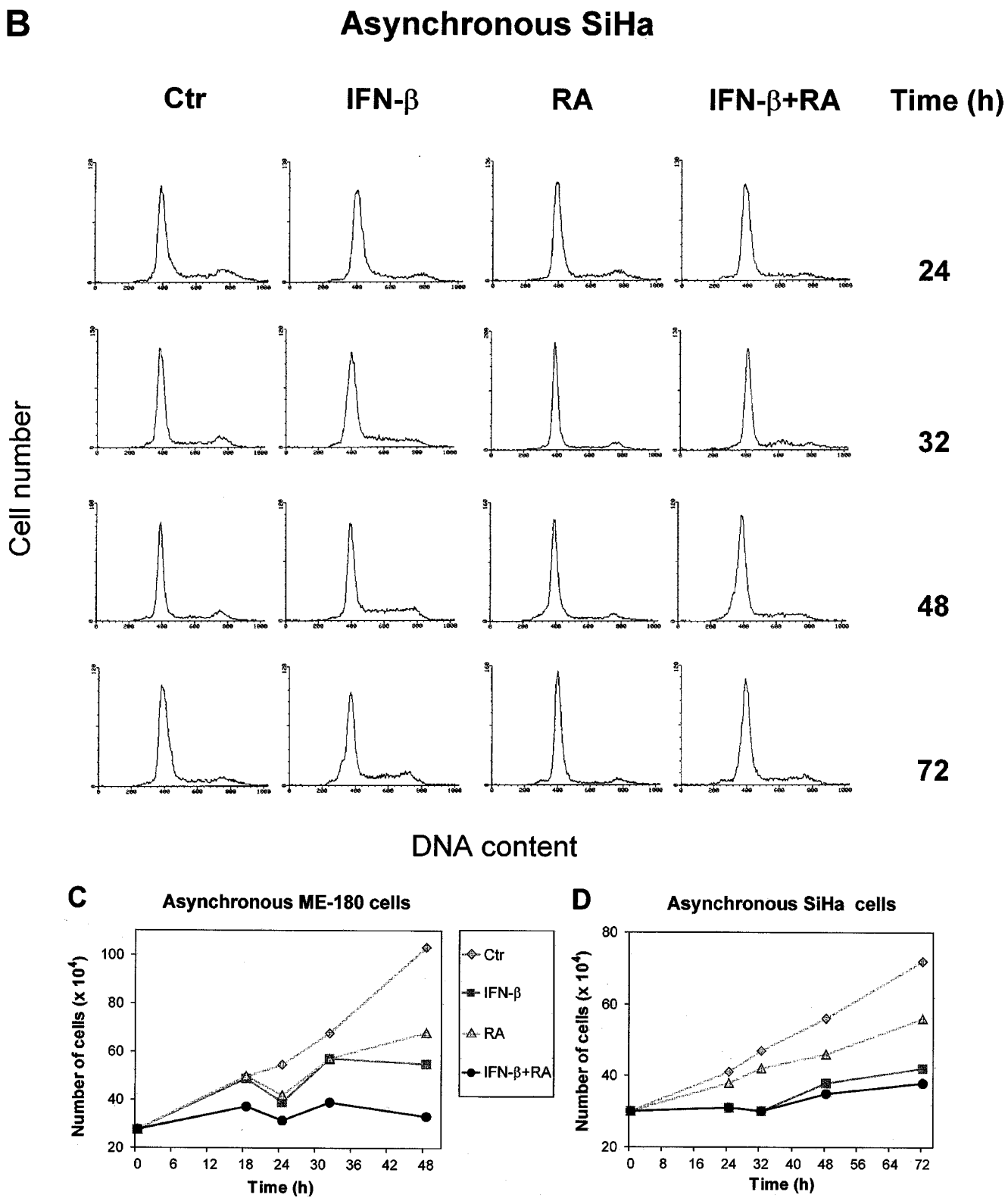


Figure 1 Cell cycle flow cytometric analysis of asynchronous ME-180 (**a**) and SiHa (**b**) cells treated with IFN-β (50 IU/ml), RA (10^{-6} M), their combination, or DMSO alone (control) for the indicated times. DNA profiles, derived from one representative experiment, show cells in G_0/G_1 (2n, large peaks), S (shoulders between 2n and 4n), or G_2/M (4n, small peaks) phase of the cell cycle. Proliferation rates in ME-180 (**c**) and SiHa (**d**) asynchronous cells treated with IFN-β (50 IU/ml), RA (10^{-6} M), their combination, or DMSO alone (control) for the indicated times. Proliferation was determined by counting adherent viable cells from parallel set of triplicate samples. The reported values are averages obtained for each time point

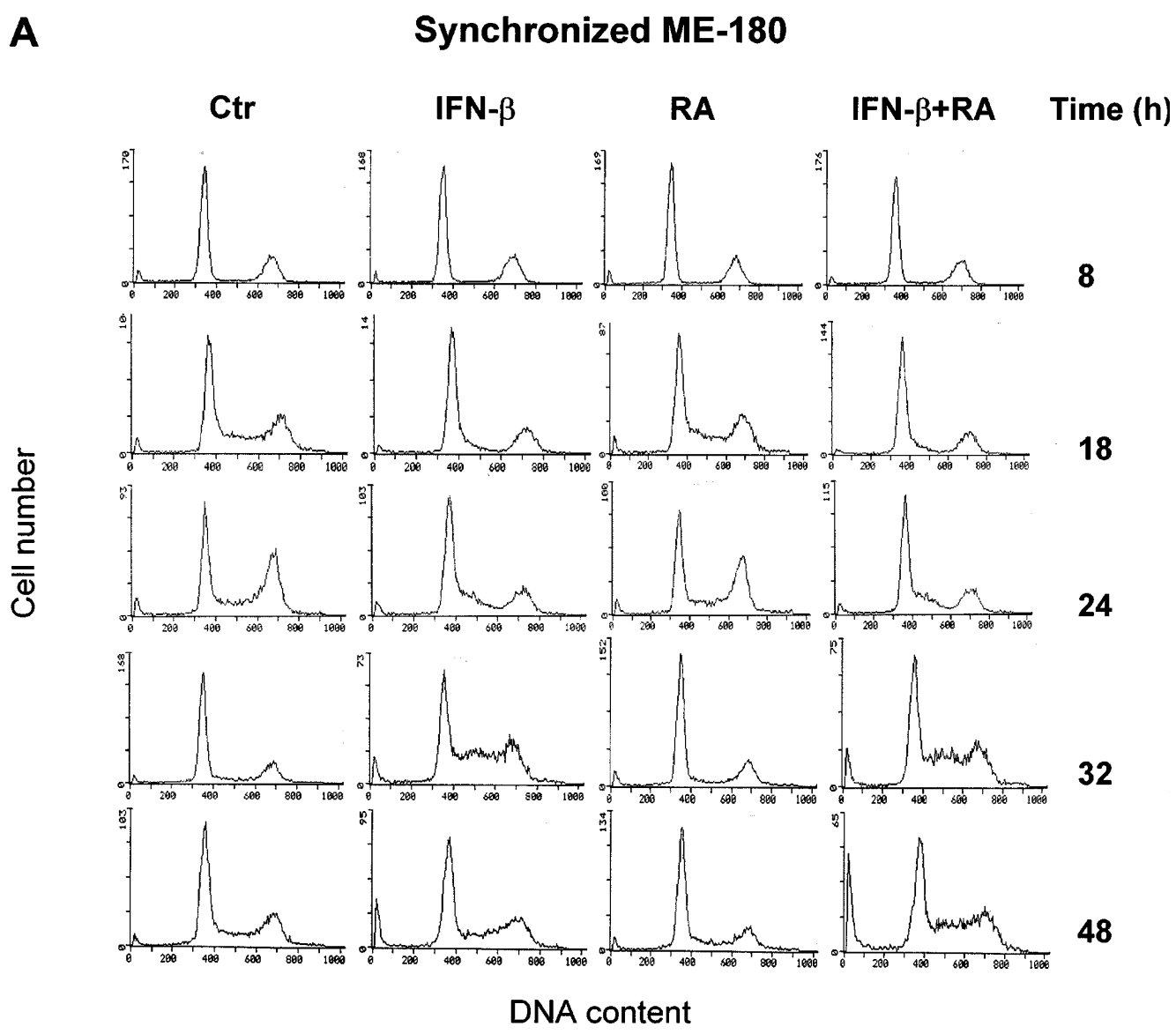
32 h in SiHa cells and reached the upper levels after 32 h and 48 h respectively. In both cellular systems this phenomenon was associated with marked growth inhibition (Figure 1c,d).

Cell cycle analysis of RA treated samples (Figure 1a,b) indicated that this agent displays a different effect with respect to IFN-β on cell cycle progression, inducing a slight increase in G_1 populations. Interestingly, in combined treatments RA increased the S phase accumulation observed with IFN-β alone in ME-180 cells whereas appeared to counteract IFN-β effect on cell cycle distribution in SiHa. As expected, the RA enhancing effect on S phase slowing down observed in ME-180 cells was coupled to its ability to highly inhibit proliferation (Figure 1c,d) in these cells with respect to SiHa cell line.

To further characterize the S phase alteration induced by IFN-β, we repeated the above experiments in ME-180 and SiHa populations synchronized in mitosis by nocodazole treatment (see Materials and methods). Synchronization was verified by FACS analysis and cells were treated 3 h after release. As shown in Figure 2a,b, all populations appeared to normally transit through G_1 phase following release from nocodazole, independently of IFN-β or RA treatment (time point of 8 h). Nevertheless, IFN-β treated cells, with respect to control and RA cells, appeared to enter and progress slowly through the S phase, accumulating strongly in it. According to the observations in asynchronous populations, in combined treatment RA potentiated the S phase lengthening due to IFN-β in ME-180, whereas in SiHa cells the addition of RA partially normalized cell cycle profile with respect to IFN-β alone, as cells progressed through the S phase.

RA added to cells as single agent did not seem to condition G_1/S transition and S phase progression following synchronization, whereas a small increase in G_1 population was revealed as cells transited through a second G_1 phase with respect to nocodazole block. The kinetics of this event suggests that RA-induced G_1 accumulation could be mediated by mechanisms which require lengthy treatments. Also in this case IFN-β/RA-induced cell cycle alterations correlate with growth inhibition (Figure 2c,d).

Taken together these results indicate that the antiproliferative effect of IFN-β on SCC cells is mediated by a specific dramatic lengthening of S



phase, that also appears to govern the synergistic action of IFN-β and RA in ME-180 cells. Since our previous studies have shown that type I IFN treatment cause cellular death and apoptosis in these cell lines (Giandomenico, 1997, 1998; Matarrese *et al*., 1998) we speculate that S phase lengthening could

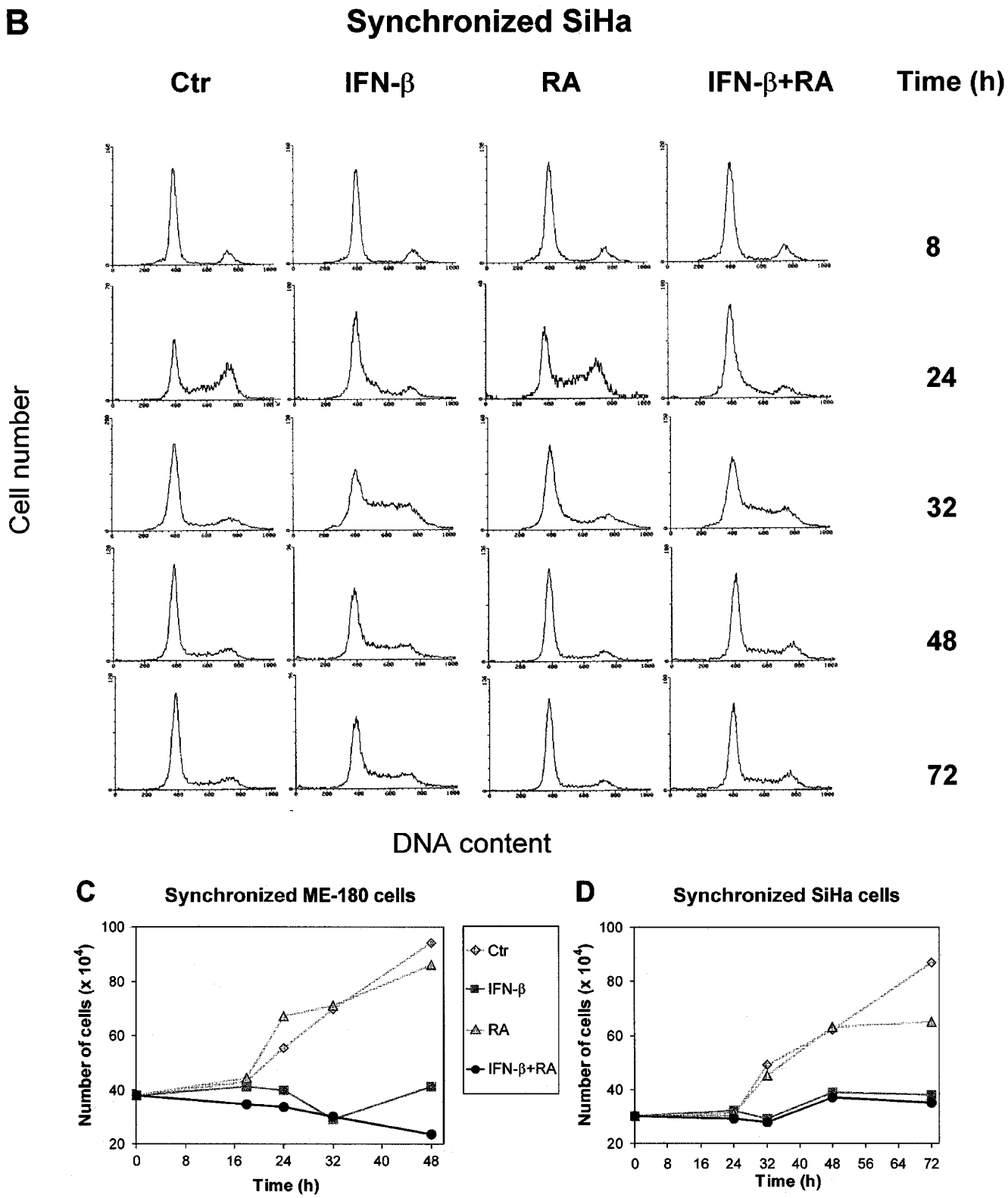


Figure 2 Cell cycle flow cytometric analysis of synchronized ME-180 (**a**) and SiHa (**b**) cells treated with IFN-β (50 IU/ml), RA (10^{-6} M), their combinations, or DMSO alone (control) for the indicated times. Cells were synchronized at G_2/M by nocodazole (0.07 μg/ml) and treated 3 h after release from nocodazole block. DNA profiles, derived from one representative experiment, show cells in G_0/G_1 (2n, large peaks), S (shoulders between 2n and 4n), or G_2/M (4n, small peaks) phase of the cell cycle. Proliferation rates in synchronized ME-180 (**c**) and SiHa (**d**) cells treated with IFN-β (50 IU/ml), RA (10^{-6} M), their combinations, or DMSO alone (control) for the indicated times. Cells were synchronized at G_2/M by nocodazole (0.07 μg/ml) and treated 3 h after release from nocodazole block. Proliferation was determined by counting adherent viable cells from parallel sets of triplicate samples. The reported values are averages obtained for each time point

reflect a disturbed cell replication leading to cellular death.

IFN-β affects DNA synthesis independently of G_1/S transit

To assess if IFN-β affects S phase progression in cells that had already passed the G_1/S transition, we synchronized ME-180 and SiHa populations in early S phase by exposing them to the DNA synthesis inhibitor aphidicolin. Immediately after release from aphidicolin block, cells were treated with 50 IU/ml IFN-β, 10^{-6} M RA and their combination. Parallel sets of samples were pulse labeled with [^{3}H]-thymidine or BrdU at several time points to determine the exact percentages of S phase cells and DNA synthesis rates.

BrdU immunofluorescence (Figure 3a,b) indicated that comparable amounts of cells entered S phase

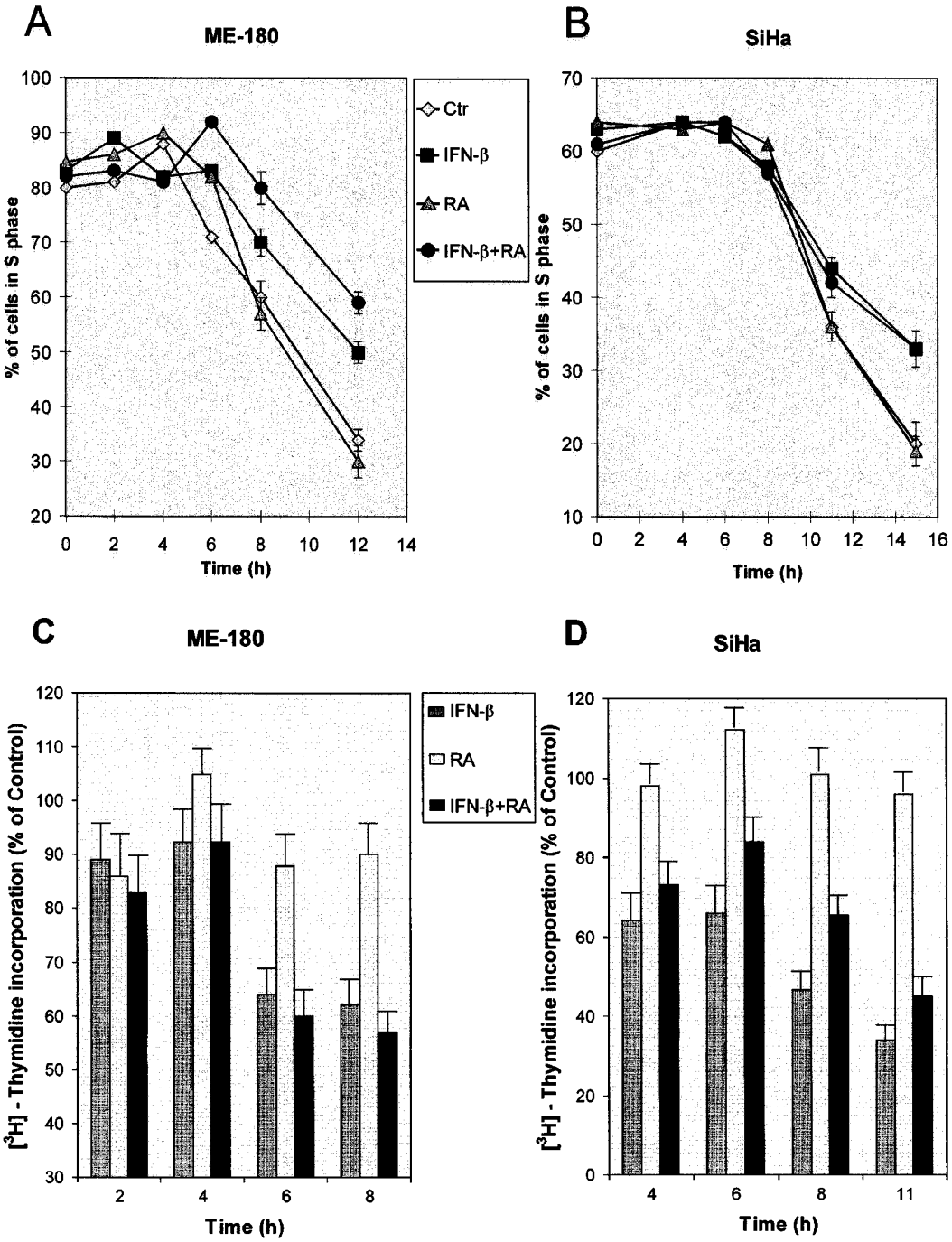


Figure 3 S phase duration in ME-180 (**a**) and SiHa (**b**) cell lines. Cells were treated with aphidicolin to arrest them at the G_1/S boundary and then released in growth media containing IFN-β (50 IU/ml), RA (10^{-6} M), their combination, or DMSO alone (control). The percentage of cells in S phase revealed by BrdU incorporation versus time after release from aphidicolin block has been reported. Shown are average values of results obtained in two independent experiments performed in quadruplicate. Bars represent standard deviation. DNA synthesis rates in S phase synchronized ME-180 (**c**) and SiHa (**d**) cells treated with IFN-β (50 IU/ml), RA (10^{-6} M), their combination, or DMSO alone (control). Cells were arrested at G_1/S boundary by aphidicolin (0.7 μg/ml) and treated immediately after release from the DNA synthesis block. At the indicated time points, cells were in parallel pulse labeled with ^{3}H-thymidine and BrdU. DNA synthesis per cell, determined by [^{3}H]-thymidine incorporation normalized on number of nuclei in S phase obtained by BrdU staining, has been reported. The reported values are averages of quadruplicate measurement $\pm$s.d.

but S phase duration appeared significantly prolonged in IFN-β treated cells. As expected, RA treated samples did not substantially differ from controls. DNA synthesis per cell (Figure 3c,d), obtained normalizing [^{3}H]-thymidine incorporation on the number of BrdU positive cells, showed that in both cell lines IFN-β was able to significantly reduce in a few hours DNA replication rate. This phenomenon increased in the next hours according to a significant delay in S phase completion. DNA synthesis rates and BrdU cellular staining also showed that at late time points RA seemed to potentiate IFN-β action in ME-180 cells but not in SiHa. These results were confirmed by DNA FACS analysis (data not shown) and demonstrate that IFN-β is able to slow S phase progression even in cells that have already passed G_1/S restriction point. Moreover, the effect on DNA synthesis is detectable within a few hours of IFN-β treatment and appears to be time dependent.

IFN-β and RA up-regulate PML expression

Since PML, among the genes regulated by IFN, was shown to have growth suppressing effect and cell cycle regulatory properties (Koken *et al*., 1995; Chelbi-Alix *et al*., 1998; Le *et al*., 1998) we investigated the molecular events associated with the observed S phase alteration due to IFN-β by analysing the expression of PML protein in ME-180 and SiHa cells treated with 50 IU/ml IFN-β, 10^{-6} M RA and their combination for several time points.

High levels of PML induced expression were observed by Western blotting (Figure 4). The visualized different bands are likely to be isoforms of PML protein derived from the 16 potential alternative splicing events proposed for the primary transcript of the unique gene (Fagioli *et al*., 1992). In addition, post transcriptional SUMO-1 modification via progressive addition of 15 kDa subunits (Müller *et al*., 1998; Kamitani *et al*., 1998; Sternsdorf *et al*., 1997), described for PML proteins, could account for the results obtained in this analysis. In ME-180 cells (Figure 4a) treated with IFN-β a clear increased expression of the major PML isoform, with an apparent MW of about 120 kDa, was detectable already at 6 h of treatment, growing up to 32–48 h of treatment (lanes 2, 6, 10, 14, 18). RA is able to affect slightly the expression of this isoform but starting after 24–32 h of treatment (lanes 11, 15). At every time, combined treatment showed a more evident 120 kDa signal (lanes 4, 8, 12, 16, 20). Other isoforms were also detected with a different kinetic of induction. In particular, a doublet at about 100 kDa appears to be specifically increased after 12 h of IFN-β or IFN-β/RA treatment up to 48 h. A faint signal at about 80 kDa appears to be induced late by IFN and IFN/RA treatment. Also a 70 kDa protein appears to be increased starting from 12–24 h in the same conditions.

In SiHa cell line (Figure 4b) the 120 kDa form, as well as the 100 kDa form, showed an induction similar to that observed in ME-180 cells. On the other hand, the 70 and 80 kDa isoforms appeared highly and early induced in this cell line starting from 6 and 12 h respectively.

In both cell lines PML upregulation was always associated with an increase in NBs size and number (data not shown).

On the basis of this evidence, quantitative evaluation of PML expression was also performed by flow-cytometric analyses in asynchronous and aphidicholin S phase-synchronized cells in order to assess whether modulation of PML expression could occur *per se* during cell cycle progression. No significant quantitative alteration in PML expression was detectable by FACS analysis of untreated cells at various cell cycle time points indicating a cell cycle-independent basal expression of the protein (data not shown). These results indicate that PML upregulation is specifically due to IFN-β treatment and correlates with the observed effect on S phase progression.

Overexpression of the PML-3 isoform reproduces IFN-β-induced S phase lengthening

The PML expression pattern described above, together with the properties ascribed to this growth suppressor gene, strongly suggested that PML protein up-regulation could account for the S phase lengthening by IFN-β in the two SCC lines. To assess this hypothesis we infected ME-180 cells with the PML-PINCO recombinant retrovirus carrying the PML-3 cDNA under the control of the 5′ LTR, since among the several PML isoforms, PML-3 seems to bind pRB with highest affinity (Alcalay *et al*., 1998). PINCO retroviral vector constitutively expresses the green fluorescence protein (GFP) as a selectable marker. The PINCO empty vector was utilized to generate control populations. Infected cells, referred to as ME-180 PINCO and ME-180 PML-PINCO, were analysed for cell cycle distribution at several time points after infection. PML-3 expression was in parallel detected on total populations by Western blot analysis.

FACS analysis of GFP expression relative to multiple independent experiments indicated that about 90% of cells resulted transduced at times ranged between 48 and 63 h after infection. Corresponding cell cycle distribution of total populations showed an increase of S phase cells about 48 h after infection in PML-3 overexpressing cells with respect to PINCO infected cells (Figure 5a).

To better determine if the cell cycle profile modification could reflect, as for the IFN-β treatments, a lengthened progression throughout the S phase, we synchronized ME-180 PINCO and ME-180 PML-PINCO cells at G_1/S boundary by aphidicolin and analysed cell cycle progression 5 and 7 h after release from DNA replication arrest. As shown in Figure 5b, DNA content profiles of PINCO cells were comparable to that of uninfected cells whereas the distribution of PML-3 expressing cells through the S phase appeared strongly delayed.

We performed the same experiments in SiHa cells, which resulted in less sensitivity to infection by PINCO recombinant retrovirus. In spite of low transduction efficiency we evidenced in selected GFP positive PML-PINCO asynchronous cells an accumulation in S phase about 77 h after infection (data not shown).

Western blot analysis of PML expression in PINCO and PML-PINCO total infected populations (Figure 4, lanes 21, 22, 25, 26) showed that in both

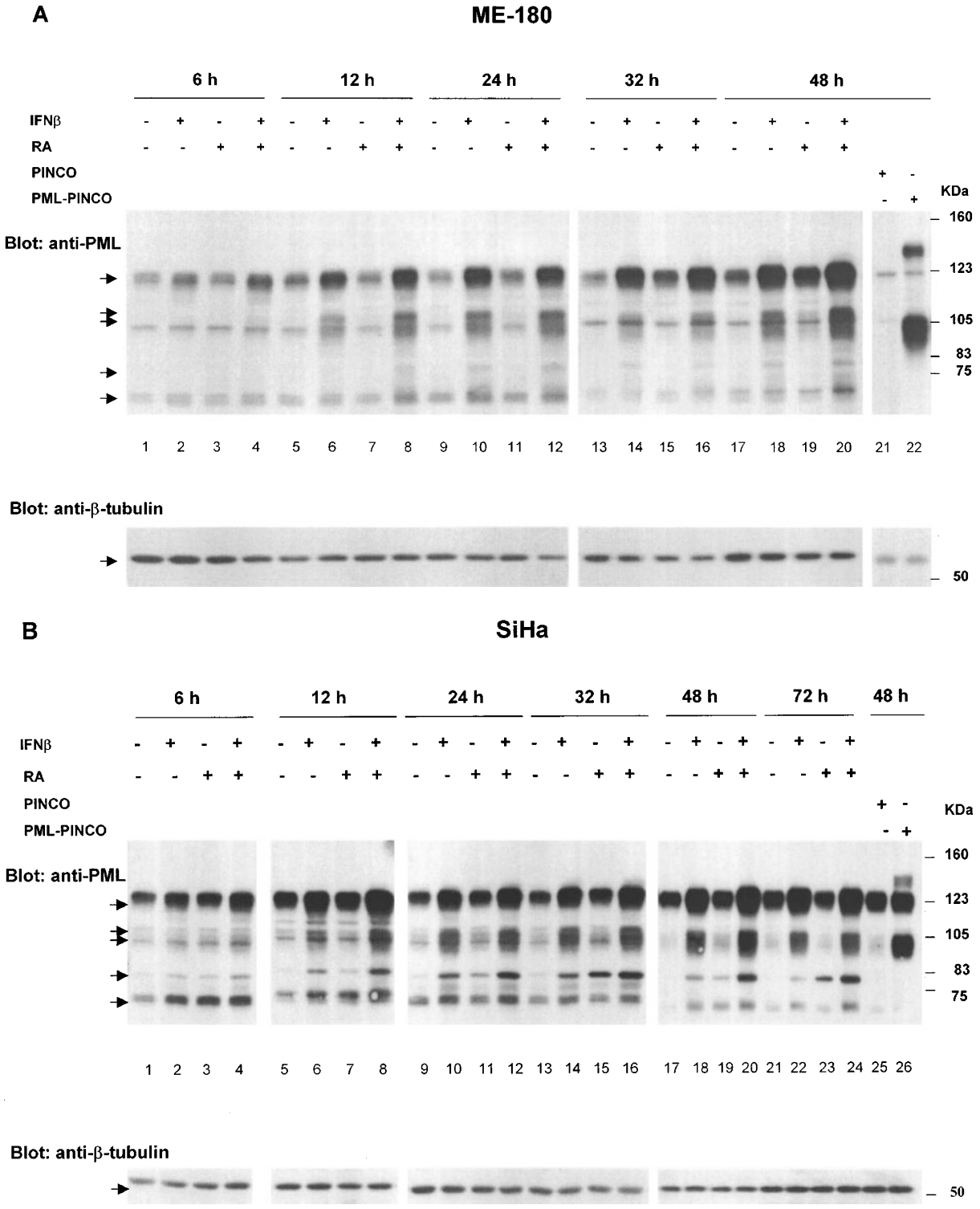


Figure 4 Western blot analysis of PML expression in ME-180 (**a**) and SiHa (**b**) cells treated with IFN-β (50 IU/ml), RA (10^{-6} M), their combination, or DMSO alone (control) for the indicated times. PINCO and PML-PINCO infected ME-180 cells (lanes 21–22) were also analysed for PML expression 48 h post-infection. Whole cell extracts were resolved on 7% SDS–PAGE and transferred onto PVDF membrane. Immunoblotting was performed with an anti-PML rabbit polyclonal antibody, followed by an anti-β tubulin mouse antibody

cell lines exogenous PML-3 migrates as two bands of about 100 and 130 kDa. PML-3 expression was observed up to about 80 h post-infection (data not shown). It is noteworthy that the endogenous PML isoform of about 100 kDa is highly up-regulated by IFN-β treatment. These results strongly suggest that PML protein exerts a pivotal role in IFN-β induced S phase lengthening.

Discussion

Among the various biological activities of IFNs, antiproliferative and pro-apoptotic activity has attracted a great deal of attention. The basic mechanisms by which IFNs affect cell cycle progression are being elucidated in several cellular systems and much evidence has indicated that imbalanced cell cycle

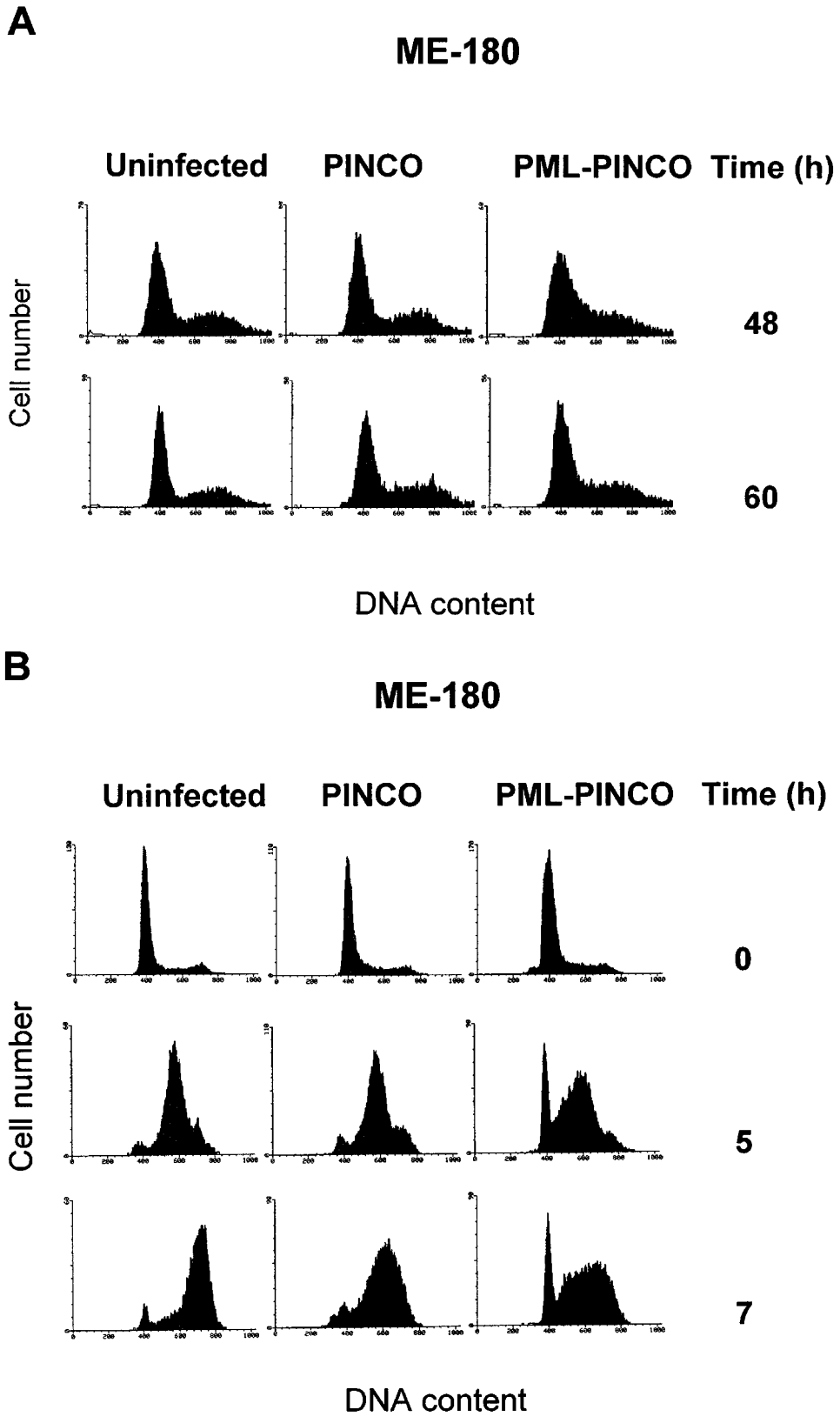


Figure 5 (**a**) Cell cycle analysis by flow cytometry of asynchronous PINCO and PML-PINCO ME-180 infected populations versus uninfected control. (**b**) Cell cycle analysis of synchronized PINCO and PML-PINCO ME-180 populations and uninfected control cells. Cells were arrested in early S phase by aphidicolin. Progression through S phase was monitored following release from the DNA synthesis block. DNA histograms derive from one representative experiment of multiple independent experiments

progression imposed by IFNs can trigger programmed cellular death.

It has been shown that PML functions as a growth and transformation suppressor when overexpressed in a variety of cell types (Mu *et al*., 1994; Koken *et al*., 1995; Le *et al*., 1998; Everett *et al*., 1999). Embryonic fibroblasts from PML$^{-/-}$ mice display higher growth rates and enhanced focus formation (Wang *et al*.,

1998a). Strikingly, the growth inhibitory activity of RA on myeloid progenitors is abrogated in the PML$^{-/-}$ background, an effect that is mirrored by the non-responsiveness to RA of the p21^{WAF1} tumor suppressor gene promoter. Analysis of PML growth suppressing mechanism has shown that, in human breast cancer cells, PML induces a G_1 cell cycle arrest associated with apoptosis, by modulating several key G_1 regulatory proteins (Le *et al*., 1998).

PML-induced apoptosis was analysed in two studies reporting different results. PML$^{-/-}$ mice and cells are protected from Fas, TNF and IFN-dependent apoptotic stimuli by a mechanism involving insufficient caspase 1 and 3 activation (Wang *et al*., 1998b) whereas overexpressed PML, in SV40 large T-transformed rat embryo fibroblasts, leads to a caspase-independent apoptosis activation (Quignon *et al*., 1998) suggesting that the PML mediated effects are exerted in a cell-type specific fashion, and/or depend critically on the expression pattern.

Thus PML possesses functions of a tumor suppressor, the activity of which appears critical in myeloid hematological malignancies as well as in solid tumors (Koken *et al*., 1995; Seeler and Dejean, 1999; Everett *et al*., 1999).

We reported previously that type I IFN and RA affect proliferation of human ME-180 and SiHa cell lines derived from squamous cervix carcinoma at different stages of differentiation (Lancillotti *et al*., 1995; Giandomenico *et al*., 1997, 1998; Matarrese *et al*., 1998; Percario *et al*., 1999). In particular, type I IFN as well as RA inhibit proliferation of ME-180 cells in a dose/time-dependent manner and a marked increase of the inhibitory effect is observed in combination treatment. Growth inhibition is associated with cell detachment and apoptosis. Type I IFN is also a potent inhibitor of SiHa cell proliferation, whereas RA affects growth poorly and not significantly enhances antiproliferative effect due to IFN in combined treatment of this cell line. In contrast to ME-180, the SiHa cell line shows flat cells strongly adhering to the substrate and forming strict intercellular relationship. Accordingly, SiHa cells are less susceptible to detachment and apoptosis induction. In addition, RA increases cell adhesion in SiHa, but not in ME-180 (Matarrese *et al*., 1998). In the present study we investigated cell cycle deregulation in these SCC lines treated with IFN-β and RA and hypothesized that the antiproliferative effect of IFN might be exerted by a cell cycle affecting function mediated by PML protein, probably via a coordinated action with cell cycle progression regulatory molecules.

In this respect, we have observed that ME-180 and SiHa cells treated with IFN-β show a significant accumulation in S phase associated with growth inhibition (Figure 1). This phenomenon reflects an S phase lengthening, as indicated by analysis of synchronized populations. In nocodazole synchronized cells, IFN-β treated populations progress more slowly than control and RA samples through the entire S phase, strongly accumulating in it (Figure 2). As a consequence, this inappropriate progression through S phase could trigger cell death. Moreover, parallel analysis of DNA synthesis rates and S phase duration, upon release from an early S phase arrest induced by aphidicolin (Figure 3), have evidenced that the IFN-β induced DNA replication slowing arises in few hours and is independent of G_1/S transition.

Interestingly, the same phenomenon seems to govern the synergistic effect of IFN-β and RA on ME-180 cells. In particular, RA addition increases the S phase lengthening due to IFN-β alone in ME-180 cells whereas it appears to counteract IFN-β effect on cell cycle distribution in SiHa (Figures 1–3). As expected the RA enhancing effect on S phase is coupled to a marked increase of cell growth inhibition in combined treatment (Figures 1 and 2).

RA added to cells as single agent displays a different effect with respect to IFN-β on cell cycle progression, inducing a slight increase in G_1 populations at late time points. The RA-induced G_1 accumulation could be mediated by mechanisms which require longer times of treatment, with respect to IFN-β, i.e. p21 CDKI induction. In fact, after several hours of RA treatment, a significant p21 CDKI overexpression has been observed (Giandomenico *et al*., 1998), possibly mediated by the RA responsive element (RARE) described in p21 promoter (Liu *et al*., 1996a).

Taken together these results indicate that the antiproliferative effect of IFN-β on SCC, in contrast to what was observed in RA-treated cells, is mediated by a specific dramatic lengthening of S phase.

Previous analyses of the mechanisms behind the ability of IFN to arrest cell growth have shown important differences in defining the type of arrest, i.e. the characteristics of the growth arrested cell populations in a variety of human transformed cells. It has been reported repeatedly (Matsuoka *et al*., 1998; Subramaniam *et al*., 1998; Sangfelt *et al*., 1999), that type I IFN is able to induce G_0/G_1 arrest, modulating the activity of G_1 cyclin/CDK-complexes, as well as to determine accumulation of cells in S phase (Qin *et al*., 1997). Also on the basis of our observations, it can be concluded that, depending on the cell types, at least two distinct cell cycle alterations could be dominant in cell cycle phenotype following IFN treatment: one involving a block in G_1 progression, probably requiring a fully functional pRB, and the other involving a failure of the cells to complete DNA replication leading to S phase prolongation.

These findings appear relevant in strategies that combine IFN treatment and conventional cancer therapies using retinoic acid as well as other tumor specific therapeutic agents. In fact, priming tumor cells towards S phase by treatment with type I IFN or IFN/RA combination (in responsive cells), might selectively render tumor cells more vulnerable to DNA-damaging agents specifically sensitizing human tumor cells to conventional cancer therapies.

Considering the possible involvement of PML protein as a mediator of the IFN-β effects on cell cycle, we have found that IFN-β strongly induces PML protein up-regulation with a kinetics overlapping the S phase alteration (Figure 4). RA is able to up-regulate PML protein only at late time points and to a lesser extent with respect to IFN-β. Moreover, among the modulated PML isoforms, a doublet of about 100 kDa appears increased by IFN-β but not by RA. It is noteworthy that the induction of PML by RA is not mediated by IFN production. In fact, we verified that RA is not able to induce type I IFN production in ME180 and SiHa cells (data not shown) in contrast to

what was previously observed in other cell lines (Pelicano *et al*., 1997, 1999).

We show that PML forced expression, performed by infection with the recombinant retrovirus PML-PINCO carrying PML-3 cDNA, is able to modify cell cycle distribution by inducing a large increase of S phase cells (Figure 5a). In addition, the analysis of the progression through the S phase of ME-180 PML transduced cells confirms that PML overexpression strongly delays the transit through the S phase (Figure 5b), as observed in IFN-β-treated ME-180 cells.

This result supports a direct involvement of the tumor suppressor PML protein in the cell cycle S phase slowing observed in IFN-β-treated squamous carcinoma cells, shedding light on a mechanism of type I IFN growth inhibition in tumor cells.

Since it has been described that PML and pRB colocalize within the NBs and that PML can form complexes with the unphosphorylated form of pRB, their interaction could regulate important processes such as differentiation, proliferation and apoptosis (Alcalay *et al*., 1998). In addition, the involvement of RB family proteins not only in G_1/S transition but also in S phase progression has been reported (Chew *et al*., 1998), leading us to hypothesize that an interaction between PML and RB family proteins may underlie the observed S phase slowing. On the other hand ME-180, as well as SiHa, are HPV-positive cells expressing E7 oncoprotein able to bind pRB and related proteins within the pocket region also involved in PML/pRB association (Hu *et al*., 1990; Kaelin *et al*., 1990; Alcalay *et al*., 1998); thus PML upregulation could also imply the alteration of pRB/E7 interaction. These hypotheses are currently under study in our laboratory.

The finding of PML involvement in growth suppression effect of type I IFN via S phase slowing in this system provides possible means by which IFN could avoid a mechanism of tumor proliferation in HPV-induced cancer and raises important questions for future investigations.

Materials and methods

Cell cultures

The human epidermoid carcinoma cell line ME-180, isolated from an omental metastasis of a rapidly spreading cervical carcinoma, was maintained in McCoy's 5a medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin/streptomycin and 2 mM glutamine. Human epidermoid carcinoma cell line SiHa, established from an undifferentiated squamous carcinoma of the cervix, was maintained in modified MEM supplemented with 10% heat-inactivated fetal bovine serum, penicillin/streptomycin, 1 mM sodium pyruvate, 2 mM glutamine and nonessential aminoacids. Both cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were grown in a humidified atmosphere of 5.5% CO_2 at 37°C. The anphotropic retrovirus packaging cells Phoenix were grown in Dulbecco medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin/streptomycin and 2 mM glutamine.

Human recombinant IFN-β (Rebif; 3×10^8 IU/mg of protein; Ares-Serono) was added to the medium from a stock solution of 10^4 IU/ml to the final concentration of 50 IU/ml. All-trans-RA (Sigma, St. Louis, MO, USA) was added to the medium from a stock solution of 10^{-3} M in DMSO to the final concentration of 10^{-6} M. Same volumes of DMSO were added to the medium of control and IFN-β treated samples.

Cell synchronization and measurement of cell proliferation

Synchronization in mitosis was obtained by culturing cells in presence of 0.07 μg/ml tubulin inhibitor-nocodazole for 16 h, followed by selective mechanical detachment of mitotic cells (mitotic shake-off). Floating cells were pelleted, washed with PBS, plated out (10^4 cells per cm^2) and treated 3 h later as described. To arrest cell cycle at G_1/S boundary, cells were seeded (10^6 cells per 100 mm dish) and treated 24 h later with 0.7 μg/ml aphidicolin (a specific DNA polymerase α inhibitor) for 18 h. Cells were then washed twice with PBS and released in fresh medium containing IFN-β, RA and their combination. To estimate cell proliferation, normal and nocodazole synchronized cells were seeded in triplicate at 10^5 cells per 35 mm dish. After 3 h, IFN-β and/or RA were added to the cultures for the indicated times. To be counted, adherent cells were detached with 0.05% trypsin – 0.02% EDTA in PBS and suspended in growth medium. Cells were counted in a hemocytometer and viability was evaluated by trypan blue exclusion.

Flow cytometry

Cells were harvested by trypsinization and washed with ice-cold PBS. To analyse cell cycle progression, cells were fixed in 70% ice-cold ethanol for at least 30 min or 4% formaldehyde in PBS for 30 min. DNA staining was performed by incubating cells at room temperature in PBS containing 0.18 mg/ml propidium iodide (PI) and 0.4 mg/ml DNase-free RNase (type 1-A). GFP expression was revealed in PINCO and PML-PINCO infected cells after fixing with 4% formaldehyde in PBS for 30 min at 4°C. In all experiments, cells were analysed on a FACScan flow cytometer (Becton & Dickinson).

[^{3}H]-thymidine and BrdU incorporation

ME-180 and SiHa cells were plated in quadruplicate at 10^5 per 35 mm dish and synchronized at G_1/S boundary by aphidicolin as described. Following the specific treatments, at the indicated time points samples were pulse labeled in parallel with 50 μM BrdU (20 min) and 5 μCi/ml [methyl-^{3}H] thymidine (79 Ci/mmol, Amersham) for 10 min. [^{3}H]-thymidine treated dishes were rinsed with ice-cold PBS and washed twice with ice-cold 5% trichloroacetic acid (TCA). Cells were then lysed at room temperature in 1 ml 0.25 M NaOH and 600 μl of the entire lysate were transferred directly into vials containing liquid scintillation cocktail (Opti-Fluor, Packard). Radioactivity was quantitated in a Packard 1500 liquid scintillation analyser. DNA synthesis per cell was evaluated normalizing [^{3}H]-thymidine incorporation on percentage of nuclei in S phase obtained by BrdU staining as described below.

Fluorescence microscopy

To determine the number of S phase nuclei in aphidicolin synchronized populations, cells were incubated with BrdU as described above. BrdU treated samples were then fixed 20 min with 95% ethanol, 5% acetic acid, treated 10 min with 1.5 M HCl and stained with an anti-BrdU monoclonal antibody (Amersham) followed by a rhodamine conjugated goat anti-mouse antibody (Cappel). Dishes were directly mounted with glycerol-PBS (1 : 1).

Western blotting

To analyse PML expression, control, IFN-β and/or RA treated cells were lysed in the following buffer: 50 mM Tris,

pH 7.4, 150 mM NaCl, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5% NP-40, 1 mM PMSF, 2 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepsatin A, 1 mM Na_3VO_3, 20 mM NaF. Total extracts were clarified by centrifugation. Protein concentration was determined (Bio-Rad Protein Assay) and 40 μg of total proteins (20 μg of ME-180 PINCO and PML-PINCO samples) were resolved on 7% SDS–PAGE and transferred onto PVDF membrane (Amersham). After blocking with 3% BSA dissolved in PBS-T (1X PBS, 0.05% Tween-20) the membrane was sequentially probed with an anti-PML rabbit polyclonal antibody (Daniel *et al*., 1993) and an anti-human β tubulin mouse IgG_1 antibody (ICN). Immune complexes were detected with horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse antiserums (Bio-Rad) followed by enhanced chemiluminescence reaction (Amersham).

Transfection and infection procedures

The EBV/retroviral vector PINCO, derived from pLZRSpBMN-Z, contains the green fluorescent protein (GFP) cDNA driven by the CMV promoter. PML-PINCO plasmid was generated by inserting the PML-3 cDNA into PINCO under the control of 5′ LTR element (Grignani *et al*., 1998). PHOENIX packaging cells were seeded at 4×10^6 cells per 175 cm^2 flask 48 h before transfection. Culture medium was then replaced with fresh medium containing 25 μM chloroquine and cells were transfected with the above plasmids by $CaPO_4$/DNA coprecipitation method (40 μg of DNA per flask). Forty-eight hours after transfection replication defective viruses were harvested from the cells, filtered through 0.45 μm Millipore filters and stored at −80°C. ME-180 and SiHa cells were plated out 24 h before infection at 10^6 cell per 75 cm^2 flask and infected at high viral-multiplicity with PINCO and PML-PINCO recombinant retroviruses in presence of 2 μg/ml polybrene.

Acknowledgments
We are particularly grateful to Marco Crescenzi and Silvia Soddu for helpful discussion of this work. We thank Roberto Gilardi and Massimo Muolo for preparing drawings, Sabrina Tocchio and Romina Tomasetto for editorial assistance. Maria Vincenza Chiantore was supported by a fellowship from FIRC. This study was supported by grants from the Consiglio Nazionale delle Ricerche 98.02927.CT04 and Progetto Nazionale MURST ex 40% 1999.

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