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Autogenous production of interferon-β switches on HLA genes during differentiation of histiocytic lymphoma U937 cells

Anat Yarden, Helen Shure-Gottlieb, Judith Chebath, Michel Revel and Adi Kimchi*

Department of Virology, The Weizmann Institute of Science, Rehovot, Israel

*To whom reprint requests should be sent
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The expression of class I HLA genes was measured during the *in vitro* differentiation of human U937 lymphoma cells towards macrophages. Following the onset of differentiation by phorbol myristate acetate the levels of cytoplasmic mRNA that hybridized with a [^{32}P]HLA-B cDNA probe increased by a factor of nine. Elevation in HLA mRNA accumulation was followed by an increase in the rate of synthesis of HLA proteins and also by a dramatic increase in class I HLA cell surface antigen expression, as shown by cytofluorimetric analysis. The elevation in HLA mRNA and surface antigens could be prevented by adding antibodies against human interferon-β (IFN-β) to the culture medium at the onset of differentiation. Interferon antiviral activity was detected in the medium of differentiated U937 cells. The same anti-IFN-β antibodies prevented the increase in (2'-5')oligo(A) synthetase activity which also takes place in differentiating U937 cells. Accumulation of the IFN-induced (2'-5')oligo(A) synthetase in U937 cells is preceded by an increase in its specific 1.6-kb mRNA as shown by hybridization to cloned (2'-5')-oligo(A) synthetase cDNA. The enzyme was preferentially found in the nuclear fraction of differentiating U937 cells. We suggest that an autogenous production of interferon-β by the differentiating cells, switches on expression of the class I HLA genes as well as that of the (2'-5')oligo(A) synthetase.
Key words: differentiation/interferon-β/HLA-A,B,C/(2'-5')oligo(A) synthetase/U937 cells

Introduction

Class I histocompatibility antigens (HLA-A,B,C in man) are 44 000 mol. wt. transmembrane glycoproteins associated with 12 000 mol. wt. β_2-microglobulin, which are expressed on the surface of nearly all adult somatic cells (Ploegh *et al.*, 1981). Expression of the class I histocompatibility antigens is developmentally regulated: though not present on embryonal carcinoma cells, the murine H_2 antigens become detectable following cell differentiation (Stern *et al.*, 1975). Very little is known about the mechanisms inducing the expression of these antigens during cell differentiation; the H_2 genes do not seem to undergo rearrangements and their expression might be controlled at the transcriptional level (Morello *et al.*, 1982). Since exposure to interferons (IFNs) increases the amount of class I and class II HLA mRNAs and proteins in a variety of cells (Fellous *et al.*, 1982; Burrone and Milstein, 1982; Basham and Merigan, 1983; Rosa *et al.*, 1983) we wondered whether IFNs could be involved in the developmentally controlled expression of HLA antigens during cell differentiation.

For these studies we used the human U937 lymphoma cell line, which was originally established from a patient with histiocytic lymphoma (Sundström and Nilsson, 1976) and is characterized by its ability to differentiate *in vitro* into cells having the morphological and functional properties of macrophages (Nilsson *et al.*, 1981; Waldrep *et al.*, 1981). We report here that human U937 cells show a strong increase in class I HLA mRNAs and surface antigens after they are induced to differentiate by treatment with phorbol myristate acetate (PMA). Addition of antibodies against purified human IFN-β to the culture medium prevents this accumulation of HLA mRNAs and proteins and also prevents a strong increase in (2'-5')oligo(A) synthetase which normally takes place in these cells during differentiation. We propose that an autocrine secretion (Sporn and Todaro, 1980) of IFN-β triggers the induction of class I HLA and (2'-5')oligo(A) synthetase gene expression in these differentiating hematopoietic cells. These results are in line with our previous report that murine Friend erythroleukemia cells spontaneously produce IFN-β and, as a result, accumulate (2'-5')oligo(A) synthetase during their differentiation towards the erythroid orthochromatic stage (Friedman-Einat *et al.*, 1982).

Results

Increase in (2'-5')oligo(A) synthetase activity during differentiation of U937 cells

Cultures of U937 cells were induced to differentiate by exposure to 50 ng/ml PMA (Nilsson *et al.*, 1981), and the level of (2'-5')oligo(A) synthetase in cytoplasmic extracts was tested at different times. Figure 1 shows that the enzyme activity was low on the first day post-induction, but reached values 40-fold higher than the basal levels on the third day. Addition of neutralizing antibodies against human IFN-β to the culture medium, together with the PMA, inhibited the increase in (2'-5')oligo(A) synthetase by 70-85% (Figure 1 and Table I). In contrast, other antibodies including antisera neutralizing IFN-α or IFN-γ were not effective and did not significantly alter the enzyme levels (Figure 1 and Table I). Thus, the elevation in (2'-5')oligo(A) synthetase following PMA treatment probably reflects the production of an extracellular IFN-β activity by the differentiating U937 cells.

The culture medium of U937 cells was tested for IFN activity at different times following induction by differentiation, by measuring the reduction of vesicular stomatitis virus cytopathic effect on human foreskin fibroblasts (Novick *et al.*, 1983). No antiviral activity was present (<4 units per ml) during the first 2 days, but titers of 5-20 international reference units per ml of IFN antiviral activity were clearly detected on day 3 after PMA. Interestingly, the IFN titer in the culture medium appears much lower than that which could be predicted from the increase in (2'-5')oligo(A) synthetase in the differentiating U937 cells, since this increase corresponded to values obtained after exposure of uninduced U937 cells to 200 units/ml of IFN-β (Table I, Figure 1). This

discrepancy could be explained either by the possible existence of cell-bound IFN, or by a difference either in the IFN or in the sensitivity of the differentiating cells to IFN. These possibilities are being presently investigated.

(2′-5′)Oligo(A) synthetase activity is also detected in the nuclei of IFN-treated cells (Nilsen *et al.*, 1982), and the nuclear enzyme may differ by its smaller size from the cytoplasmic synthetase (St. Laurent *et al.*, 1983). We examined the distribution of (2′-5′)oligo(A) synthetase activity between the two cell compartments in differentiated U937 cells and in uninduced cells treated by exogenous IFN. In the differentiated cells, the ratio between the nuclear and cytoplasmic activities was 3-fold higher than in IFN-treated undifferentiated cells (Table I). Following PMA induction the level of synthetase activity increased more strongly in the nuclear fraction of U937 cells, but with similar kinetics to those observed for the cytoplasmic enzyme in Figure 1 (data not shown). Thus, the induction of (2′-5′)oligo(A) synthetase during cell differentiation, resulting from the autogenous production of IFN-β is characterized by a preferential increase in the nuclear enzyme over the cytoplasmic activity.

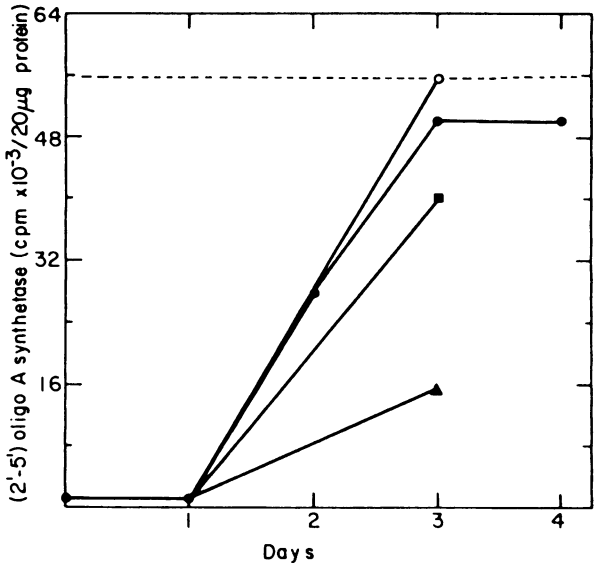

Fig. 1. Effect of antisera to human IFN-α, β and γ on the increase in (2′-5′)oligo(A) synthetase during differentiation of U937 cells. Differentiation was initiated by exposure of 4 x 10^5 cells/ml to PMA (50 ng/ml) (Sigma). The different antisera were added to the culture medium immediately following the addition of the inducer of differentiation at a final dilution sufficient to neutralize 100 units/ml of the corresponding IFN. At the indicated time after PMA, cell extracts were prepared (1 – 2 x 10^6 cells/100 μl) and samples were tested for (2′-5′)oligo(A) synthetase activity as described in Materials and methods. ●, no antiserum; ▲, antiserum to IFN-β; ■, antiserum to IFN-α; ○, antiserum to IFN-γ. The dotted line represents the values of synthetase activity in undifferentiated cells treated for 24 h with IFN-β (200 U/ml).

We measured the amount of (2′-5′)oligo(A) synthetase E mRNA by hybridization of the cloned E cDNA probe recently isolated (Merlin *et al.*, 1983), to Northern electrophoretic blots of poly(A)$^+$ RNA extracted from U937 at different times after induction of differentiation. As shown in Figure 2A, a strong induction of the (2′-5′)oligo(A) synthetase 1.6-kb E mRNA, as detected in other cells (Merlin *et al.*, 1983), is seen on days 2 and 3 after PMA treatment, in contrast to the virtual absence of (2′-5′)oligo(A) synthetase E mRNA in uninduced U937 cells (lane 4, Figure 2A) and on day 1 after PMA. The kinetics of E mRNA accumulation compared with enzyme accumulation (Figure 1) support the conclusion that it is the expression of the (2′-5′)oligo(A) synthetase gene which is activated during the differentiation of U937 cells.

Class I HLA mRNA accumulation during differentiation of U937 cells

Undifferentiated U937 cells do not express actively the class I HLA genes, and RNA which hybridizes to a HLA-B [^{32}P]-cDNA probe cannot be readily detected in uninduced cells on short exposures of the autoradiograms (Figure 2B, slot 4). In contrast, in poly(A)$^+$ RNA extracted from U937 cells on days 1 and 2 after PMA treatment, a strong RNA band hybridizing on nitrocellulose blots to the HLA cDNA probe, appears at 1.7 kb as expected for HLA-A,B,C mRNA (Ploegh *et al.*, 1980) (Figure 2B, slots 1,2). During the differentiation process, accumulation of class I HLA mRNA is, therefore, switched on even prior to that of the (2′-5′)-oligo(A) synthetase mRNA.

The increase in class I HLA mRNA was quantitated by the dot-blot procedure. Figure 2C shows that the maximal level of HLA-A,B,C mRNA in the differentiating U937 cells was 9-fold higher than the basal level in undifferentiating cells. To determine whether IFN is involved in the induction of the HLA-mRNA, neutralizing antiserum to IFN-β was added to the culture medium of the differentiating cells. Figure 2C shows that most of the increase in HLA-mRNA was prevented by the anti-IFN-β antibodies, as found for the increase in (2′-5′)oligo(A) synthetase activity (Figure 1). Induction of HLA requires less IFN-β than induction of the (2′-5′)oligo(A) synthetase (Fellous *et al.*, 1982), which may explain why the HLA-mRNA, appears before the synthetase mRNA in the U937 cells, and much before free IFN is detected in the culture medium of the differentiating cells.

Table I. (2′-5′)Oligo(A) synthetase in differentiating U937 cells

(2′-5′)Oligo(A) synthetase	Undifferentiated cells		Differentiated cells (3 days post-PMA[b])			
	No IFN	+IFN-β[a]	PMA	PMA +anti-IFN-β	PMA +anti-IFN-α	PMA +anti-IFN-γ
Activity in cytoplasmic extracts[c] (c.p.m.)	3250	57 770	50 250	7300	43 900	45 140
Ratio nuclear:cytoplasmic activity	1.22	0.99	3.02	–	–	–

[a]Exponentially growing U937 cells (2 x 10^5 cells/ml) were treated for 24 h with human IFN-β (200 U/ml). The nuclear and cytoplasmic fractions were prepared and tested for (2′-5′)oligo(A) synthetase activity as in Materials and methods.
[b]Differentiation was induced as described in the legend to Figure 1.
[c][^{32}P](2′-5′)Oligo(A) synthesized in c.p.m./20 μg protein of cytoplasmic or nuclear extracts as described in Materials and methods.

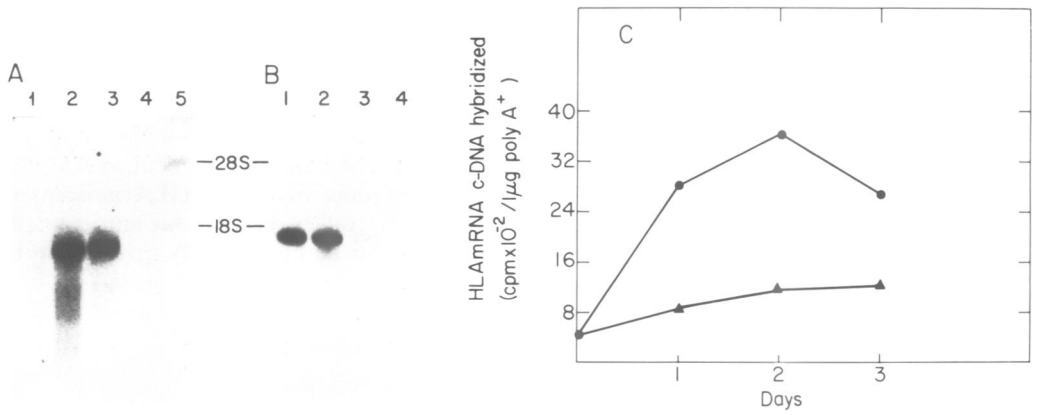

Fig. 2. Increase in HLA mRNA and (2′-5′)oligo(A) synthetase mRNA during differentiation of U937 cells. Cytoplasmic poly(A)$^+$ RNA was prepared at different time intervals following exposure of U937 cells (4 x 10^5 cells/ml) to 50 ng/ml PMA, as described in Materials and methods and samples were Northern blotted following gel electrophoresis and hybridized to ^{32}P-radiolabeled probes. **(A)** Hybridization with (2′-5′)oligo(A) synthetase cDNA probe (Merlin *et al.*, 1983); autoradiographic exposure time was 3 days with intensifying screen at −70°C. **(B)** The same blot was hybridized again with HLA-B cDNA (Sood *et al.*, 1981); autoradiographic exposure time was 3 h. **Lanes 1,2,3:** poly(A)$^+$ RNA from cells at 24, 48 and 72 h post-PMA induction, respectively; **lane 4:** poly(A)$^+$ RNA from control uninduced cells; **lane 5:** ribosomal [^{32}P]RNA used as marker. **(C)** In another experiment, U937 cells were induced to differentiate by PMA in the presence (▲) or absence (●) of antiserum to human IFN-β as in Figure 1, and the frequency of HLA mRNA in poly(A)$^+$ RNA was quantitated by the dot blot hybridization technique as described in Materials and methods.

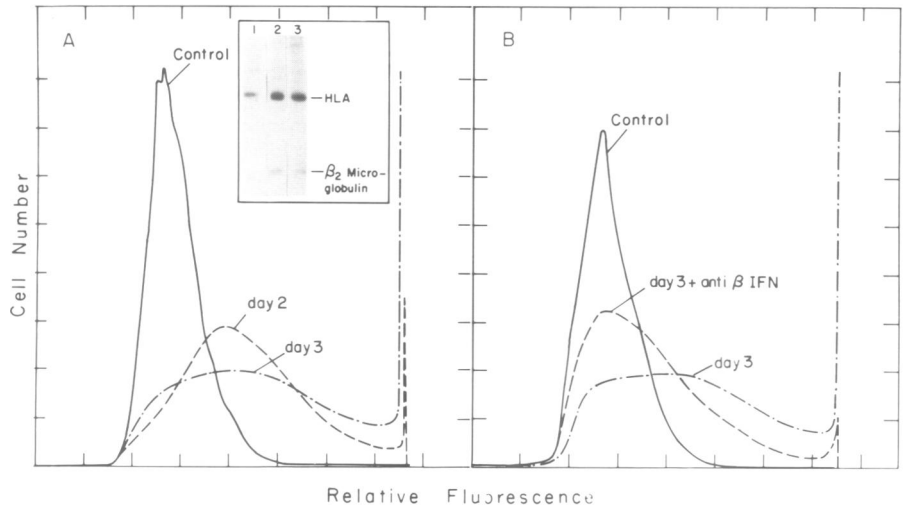

Fig. 3. Changes in the level of HLA proteins during U937 cell differentiation: rate of synthesis and accumulation on the cell surface. U937 cells were induced to differentiate by PMA as in Figure 1. An exponential culture at 5 x 10^5 cells/ml was used to determine the basal levels in control uninduced cells. At the indicated times, samples of 4 x 10^6 cells were pulse-labeled with [^{35}S]methionine to determine the rate of HLA nd β_2-microglobulin synthesis as illustrated in the insert of **A. Lanes 1,2,3:** control cells uninduced and days 2 and 3 post-PMA induction, respectively. In another set of 2−4 x 10^6 cells, the levels of HLA surface antigens on different days after PMA were measured by direct immunofluorescence in the FACS **(A)**. In **(B)** antiserum to IFN-β was added to the culture medium of differentiating cells as in Figure 1. **A,** (——), control uninduced cells; (------), 2 days following PMA induction; (—·—·), 3 days following PMA induction. **B,** (——), control uninduced cells; (—·—·), (------), 3 days following PMA in the absence and presence of antiserum to IFN-β, respectively.

Increase in the rate of synthesis of class I HLA proteins and their accumulation on the cell surface during U937 cell differentiation

The induction of HLA-mRNA during differentiation of U937 cells is followed by an increase in the rate of synthesis of HLA-A,B,C chains (mol. wt. 44 000) and of β_2-microglobulin (mol. wt. 12 000) together with an increase in the amount of these HLA antigens exposed on the surface of the cells. We labeled U937 cells with [^{35}S]methionine at different time intervals after PMA, and immunoprecipitated the cytoplasmic β_2-microglobulin, together with its associated HLA-A,B,C chains, using polyclonal antibodies against β_2-microglobulin. The rate of synthesis of both HLA protein components markedly increased on day 1 and 2 after the onset of PMA-induced differentiation (Figure 3, insert). These changes are also expressed on the cell surface as shown by fluorescence-activated cell sorter (FACS) analysis of the cells stained by indirect immunofluorescence with monoclonal antibodies against class I HLA (Figure 3A). The U937 cell population showed a marked increase in the surface class I HLA antigens on day 2 after PMA, which continued on day 3. In line with what we observed for HLA mRNA, the addition of anti-IFN-β antibodies at the onset of differentiation inhibited most of the increase in cell surface class I HLA antigens observed at day 3 (Figure 3B).

Discussion

This work shows that the expression of class I HLA genes is switched on during the differentiation of U937 lymphoma cells towards macrophage-like cells. From day 1 to 3 after in-

duction to differentiation by PMA, HLA-A,B,C mRNA accumulates, HLA and β_2-microglobulin protein synthesis increases and the antigens accumulate on the cell surface. These events are inhibited if neutralizing antibodies against human IFN-β are added to the culture medium of the U937 cells. Other antibodies have no such effects. The induction of HLA gene expression appears, therefore, to occur in response to extracellular IFN molecules, which would be secreted by the differentiating cells. In support of this concept, IFN antiviral activity can be detected, albeit at low levels, in the medium of cultures after 2–3 days of PMA induction. Furthermore, the expression of another IFN-dependent gene, coding for the (2′-5′)oligo(A) synthetase, is also activated in the differentiating U937 cells and inhibited if anti-IFN-β is added. We have verified that the secretion of IFN is not due to the PMA inducer itself, and is observed also if differentiation of the U937 cells is induced by IFN-free lymphokine preparations (unpublished results). Measure of the cell surface HLA antigens in the FACS demonstrates that the increase in HLA antigens occurs in the majority of the cells in the population, suggesting that almost all the cells respond to the IFN produced. We propose that IFN functions here in an autocrine secretion mechanism. The autocrine mechanism, in which cells secrete substances to which they actively respond through their own extracellular receptors, was proposed previously for transforming growth factors (Sporn and Todaro, 1980).

Several differences in the pattern of (2′-5′)oligo(A) synthetase induction are apparent if one compares differentiating U937 cells and cells treated by exogenous IFN. In most cells, exogenous IFN produces a transient induction of the (2′-5′)-oligo(A) synthetase mRNA lasting <24 h (Merlin *et al.*, 1983), while high levels of this mRNA are maintained in the differentiating U937 cells for at least 48 h. Furthermore, the (2′-5′)oligo(A) synthetase produced during the differentiation of U937 cells is preferentially located in the nucleus, in contrast to the enzyme induced by IFN in the undifferentiated cells. Large and small forms of the synthetase have been observed, which may be coded for by different mRNAs (Revel *et al.*, 1981; St. Laurent *et al.*, 1983; Merlin *et al.*, 1983). The 1.6-kb RNA, hybridizing to the (2′-5′)oligo(A) synthetase cDNA and which is strongly induced in the differentiating U937 cells, could code for the smaller 30 000 mol. wt. form of the enzyme which has been reported to be mainly nuclear (St. Laurent *et al.*, 1983). The possible role of the nuclear synthetase in the regulation of cell functions (Nilsen *et al.*, 1982) is still uncertain.

We have previously reported that the secretion of IFN-β and a large increase in (2′-5′)oligo(A) synthetase is also observed during the differentiation of Friend erythroleukemia cells, and that the IFN produced differs in some properties from the viral-induced murine IFN-β (Friedman-Einat *et al.*, 1982). We do not know yet whether the human IFN-β secreted by the differentiating U937 cells is identical to the poly(rI):(rC)-induced fibroblastic IFN-β_1. Neutralization of this IFN does not prevent morphological differentiation in U937 cells (unpublished data). Nevertheless, IFN-β could participate by its known anti-growth effect (Kimchi *et al.*, 1981a, 1981b; Creasy *et al.*, 1983) in the commitment to terminal cell division which occurs during the differentiation program. Our data on U937 and Friend cells, together with previous observations that mature peripheral T lymphocytes (Kimchi, 1981) and rabbit reticulocytes (Hovanessian and Kerr, 1978) contain high levels of (2′-5′)oligo(A) synthetase, suggest that production of some IFNs is part of the normal differentiation process of these hematopoietic cells.

IFNs are the only factors presently known to modulate the synthesis and cell surface expression of HLA antigens. In as much as class I histocompatibility antigens are also markers of cell differentiation (Stern *et al.*, 1975), the role of IFN-β in the appearance of class I HLA antigens on the surface of U937 cells would argue that the autocrine production of IFN-β could also be more deeply involved in the differentiation program.

Materials and methods

Antisera and interferons

Anti-human IFN-β serum was prepared in rabbits injected with purified IFN-β_1 obtained from poly(rI):(rC)-induced foreskin cells and purified by chromatography on Cibacron Blue-Sepharose columns (Novick *et al.*, 1983; Knight and Fahey, 1981). The neutralizing titer against IFN-β_1 was 20 000 units/ml. This antiserum immunoprecipitated only the 20 000 mol. wt. band of IFN-β_1 in a [^{35}S]methionine-labeled Blue-Sepharose fraction used as immunogen (Zilberstein *et al.*, unpublished results). Antiserum to human IFN-α (10 000 units/ml) (G-026-502-568) was kindly provided by the NIAID. Antiserum to human IFN-γ (10 000 units/ml) was kindly provided by H.Johnson (Texas). Human IFN-β_1 (4 x 10^8 units/mg protein) was purified by Blue-Sepharose and h.p.l.c. as described before (Novick *et al.*, 1983). The HLA-B cDNA clone was a kind gift from S.M.Weissman (Sood *et al.*, 1981). The (2′-5′)oligo(A) synthetase E_1 cDNA clone was described elsewhere (Merlin *et al.*, 1983).

Induction of differentiation in U937 cells

The U937 cell line (Sundström and Nilsson, 1976) was grown at 37°C in a humidified atmosphere of 5% CO_2 in air in RPMI 1640 (Gibco) supplemented with 10% heat-inactivated fetal calf serum (FCS). Differentiation was initiated by exposing 4 x 10^5 cells/ml to 50 ng/ml PMA (Sigma). Differentiation towards macrophage-like cells was followed for 1–4 days.

(2′-5′)Oligo(A) synthetase assay in crude extracts

Crude extracts were prepared in Nonidet P-40 (NP-40, 1–2 x 10^6 cells/100 μl) as described before (Kimchi, 1981) for the control undifferentiated cells which grow in suspension, or as detailed elsewhere for the differentiated cells which adhere to the plastic dishes (Kimchi *et al.*, 1979). Samples of 20 μg protein of the 10 000 *g* supernatant fraction were adsorbed on poly(rI):(rC)-agarose beads (P.L.Biochemicals) which were then incubated with [α-^{32}P]ATP (3 Ci/mmol; 2.5 mM) as previously detailed (Kimchi *et al.*, 1979).

Preparation of nuclear extracts

Cells were washed in 35 mM Tris-HCl pH 7.6, 140 mM KCl, 3 mM $MgCl_2$ and swollen in hypotonic buffer A (10 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 10 mM NaCl) for 10 min on ice. The cells were then homogenized in a Dounce homogenizer. The homogenate was centrifuged 10 min at 1500 *g* and the supernatant was kept as a cytoplasmic extract and was stored in liquid nitrogen. The nuclear pellet was resuspended in buffer A, layered over a 25% glycerol cushion (3 ml) and centrifuged 10 min at 1500 *g*. The pellet was resuspended in buffer A containing 0.01% Triton X-100 plus 25% glycerol and centrifuged again. Following an additional wash in buffer A, the nuclei were lysed (1.5 x 10^8 nuclei/ml) in hypertonic buffer (10 mM Tris-HCl pH 7.6, 500 mM KCl, 1 mM EDTA, 10% glycerol, 5 mM β-mercaptoethanol, 50 mM phenylmethylsulfonylfluoride) for 30 min on ice and the nuclear residue was sedimented by centrifugation at 10 000 *g*. The supernatant fraction was dialysed against buffer B (20 mM Hepes pH 7.5, 120 mM KCl, 5 mM MgCl, 1 mM dithiothreitol, 10% glycerol). This nuclear extract contained 3–5 mg protein/ml and was stored in liquid nitrogen. (2′-5′)Oligo(A) synthetase activity was assayed as described above in 20 μg of the cytoplasmic and the nuclear fractions.

RNA isolation and hybridization

1–2 x 10^8 cells were washed in phosphate-buffered saline (PBS) and extracted in buffer B containing 0.5% NP-40 and 10 mM diethylpyrocarbonate (Sigma). After 10 min on ice, the cell extracts (5 ml) were centrifuged at 10 000 *g* for 10 min, the supernatant was adjusted to 40 mM Tris-HCl pH 9, 2 mM EDTA and 0.1% SDS, and the RNA was extracted with 2 volumes of phenol/chloroform, 1:1 (v/v), and then with 1 volume of chloroform/isoamyl alcohol, 99:1. 20 μg of poly(A)$^+$ RNA obtained by purification on oligo(dT)-cellulose chromatography were subjected to electrophoresis on 1% agarose gel in 6% formaldehyde, transferred to the nitrocellulose sheets, dried and baked as described (Fellous *et al.*, 1982).

Hybridization to 10^7 c.p.m. of each of the different probes (radiolabeled by

nick-translation to a specific activity of $1-3 \times 10^8$ c.p.m./μg) was performed as detailed in Fellous *et al.* (1982). For the dot-blot hybridization, 2-fold serial dilutions of each preparation of poly(A)$^+$ RNA were spotted on nitrocellulose sheets (from 3 μg to 0.04 μg in 3 μl). The sheets were dried, baked and hybridized to HLA [^{32}P]cDNA probe as above. Following autoradiography the spots were cut and counted and the amount of radioactivity hybridized to 1 μg of poly(A)$^+$ RNA was calculated.

Measure of protein bio-synthesis

[^{35}S]Methionine incorporation was done by incubating 4 x 10^6 cells in methionine-free medium containing 50 μCi/ml [^{35}S]methionine (Amersham 800 Ci/mmol), for 1 h. The cells were washed in cold PBS, extracted with 0.5% NP-40 in 10 mM Tris-HCl pH 7.6, 150 mM NaCl, 1 mM EDTA (solution I) and clarified for 10 min at 10 000 *g*. Fractions of cell lysates containing 2 x 10^6 trichloroacetic acid-precipitable c.p.m. were incubated for 1 h at 4°C with polyclonal antibodies against β_2-microglobulin (Dako). Protein A-Sepharose CL-4B beads (Pharmacia) were added for 1 h at 4°C with stirring. The beads were washed repeatedly in solution I, solubilized in SDS and run on a 10–20% polyacrylamide gradient gel. Autoradiography was done for 24 h following fluorography of the gels.

Cytofluorimetric analysis

Indirect fluorescence staining was performed on cells which were washed three times with RPMI 1640 with 2% heat-inactivated FCS (the differentiated cells were detached from plates using PBS with 0.03% EDTA before the washing). Cells (2 x 10^6 total) were incubated at 4°C for 45 min with monoclonal antibodies specific for HLA-A,B,C (BRL Inc.) in a volume of 50 μl. Cells were then washed three times and incubated at 4°C for 45 min, with 10 μl of (1:10 diluted) fluoresceine-isothiocyanate conjugated goat-anti mouse immunoglobulin (Nordic Immunological Laboratories). Cells were washed by centrifugation through 1 ml of heat-inactivated FCS. Myeloma supernatant controls were used for every experiment to evaluate the level of autofluorescence and non-specific staining. Quantitative evaluation of immunofluorescence was performed on 40 000 cells in the FACS II (Becton-Dickinson) at 500 V. Non-specific staining was low and would have appeared at the origin of the scale (not shown).

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