Translation of lymphocyte mRNA for mouse colony stimulating factor

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ABSTRACT

Poly A⁺ RNA has been purified from phorbol myristate acetate stimulated mouse EL4 cells. Translation, by microinjection into frog oocytes, gave biologically active interleukin 2 (IL2) and colony stimulating factor for granulocytes and macrophages (GM-CSF). These two mRNAs were separated by centrifugation through linear sucrose gradients. The sedimentation coefficients for IL2 and GM-CSF mRNAs were found to be 11.5S and 8S respectively. The clonal and cellular morphologies induced by the oocyte material corresponded to low concentrations of authentic GM-CSF.

INTRODUCTION

The proliferation and differentiation *in vitro* of committed granulocyte and macrophage progenitor cells is dependent on the presence of glycoproteins known as colony stimulating factors. They stimulate the development of granulocyte and macrophage colonies in semi-solid cultures of bone marrow cells and are, probably, major regulators of granulocyte and monocyte-macrophage formation *in vivo* (1).

Colony stimulating factors are produced by a variety of tissues in mouse, including fibroblasts, macrophages, mitogen-stimulated lymphocytes, and endothelial cells (2). Factors from two murine sources have been purified to homogeneity. One of these, macrophage colony stimulating factor (M-CSF), has been purified from mouse L-cell conditioned medium (3). It is a glycoprotein with M.W. 60,000 – 70,000, which stimulates macrophage production preferentially. The other, purified from mouse lung conditioned medium (4) is a glycoprotein with M.W. 23,000 and stimulates both macrophage and granulocyte production (GM-CSF).

CSF can also be obtained from murine T lymphoma cell lines. Stimulation of a T cell hybridoma with the mitogen concanavalin A (5) or of EL4 thymoma with the tumor promoter, phorbol-12-myristate-13-acetate (PMA) (6), induced these cells to produce CSF. The MW of the thymoma produced factor was estimated to be 30,000 in native form and 23,000 after denaturation. Morphological examination of colonies developing in semi-solid culture, in the presence of this CSF, revealed macrophage, granulocyte and mixed colonies. On the basis of

these observations this activity may be very similar to GM-CSF isolated from mouse lung conditioned medium (4).

CSF belongs to a large group of soluble factors called lymphokines or cytokines. These are synthesized by activated T cells and regulate the growth and differentiation of lymphoid and other hemopoietic cells. It is obviously important to establish the molecular structures responsible for lymphokine activities. Purification to homogeneity is difficult and cannot, in principle, prove the relationship between structure and function. An important alternative line of attack is to synthesize the proteins responsible for each activity using cloned genetic material. The latter approach would also enable a study to be undertaken on the gene structure, regulation of expression, and evolutionary relationships between a series of biologically important lymphokines.

The mouse T lymphoma EL4 is a rich source of a number of lymphokines including Interleukin 2, Interleukin 3, Colony Stimulating Factor, B Cell Growth Factor (7) and B Cell Differentiation Factor (8). To clone the genes for these molecules it is important to develop an efficient system for the translation of mRNA into biologically active proteins. In vitro translation of biologically active IL2 from EL4 mRNA has recently been reported (9). In this paper we report the successful translation of mRNA from the same source into biologically active GM-CSF. We also demonstrate that the CSF and IL2 are separate entities, encoded by separate mRNAs, as recently shown for human homologues (15).

MATERIALS AND METHODS

Cells and Animals

The variant of murine T lymphoma line EL4 has been described previously (7). Typically 5×10^4 cells were suspended at 10^6 cells/ml in medium RPMI 1640, either serum-free, or containing 4% horse serum. The inducer phorbol myristate acetate (PMA) was added at 10 ng/ml, and cells were collected by centrifugation after 14 hours of incubation. Xenopus laevis were maintained in the Department of Zoology, University of Alberta.

Measurement of colony stimulating activity

The GM-CSF assay was done by a modification of techniques described previously (10, 11). Bone marrow was obtained from femurs of 8–12 week old mice (BDF₁ hybrid males) by flushing with MEM α medium (Gibco). Nucleated bone marrow cells (75,000) were suspended in 1 ml of the same medium containing 15% horse serum (Flow Laboratories), and 10 μ l (1 oocyte equivalent) of either oocyte homogenates or supernatants. Samples (in triplicate) were incubated in a humidified atmosphere at 7% CO₂, 37°C and colonies were counted on day 7. Aggregates of 50 cells or more were scored as colonies, aggregates of 10–50 cells as clusters.

Assay for IL2

As described previously (9), samples were incubated overnight with the cytotoxic T cell line MTL2.8.1 (12) which is dependent upon IL2 for growth. The supernatants were removed and replaced with fresh medium containing 25ug/ml phenylmethylsulphonylfluoride (PMSF), ^{125}I UdR (5×10^5 cpm/ml), 5 ug/ml FUDR, 100 uM inosine. After 6h, the cells were harvested and incorporated label was determined by gamma counting.

Isolation of poly A⁺ RNA

Stimulated cells (5×10^6) were lysed in 20 ml 6M guanidine hydrochloride, 0.1M potassium acetate, 10mM 2-mercaptoethanol in a Servall homogenizer (13). RNA was precipitated by 0.5 volumes ethanol, kept at -20° for at least 8h. The precipitate was dissolved in 6M guanidine hydrochloride, 0.1M potassium acetate, 25 mM EDTA and precipitated with ethanol. This was repeated once more. The RNA was dissolved in 25 mM EDTA, 0.1% sodium dodecyl sulphate, made 0.3M in sodium acetate, pH 4.5, and precipitated with 2.5 volumes of ethanol at -20° overnight. The material was finally dissolved in 0.5M LiCl, 50 mM Tris.Cl, pH 7.5, 0.1% SDS, 0.2 mM EDTA, and run over oligo dT cellulose (Type T3, Collaborative Research). The poly A⁺ fraction was eluted in a similar solvent lacking LiCl. It was re-run over the column, this time including an intermediate salt wash (0.1M LiCl). The final poly A⁺ fraction (2%) was precipitated twice from 0.3M sodium acetate with 2.5 volumes ethanol at -20° .

Sucrose gradient centrifugation of RNA

Poly A⁺ RNA was dissolved in water, heated to 70° for 1 minute, quickly chilled and then made 50 mM in Tris.Cl, pH 7.5, 0.1% in lithium dodecyl sulphate, and 2 mM in EDTA. This sample was loaded onto a 10 to 28% linear sucrose gradient containing the same salts and centrifuged in an SW 50.1 rotor at 48,000 rpm for 5h at 4° . Gradient fractions were precipitated twice from 0.3M sodium acetate with ethanol. The final precipitates were dissolved in water for in vitro translation.

Microinjection of oocytes

The mRNA fractions were injected (normally 30 nL) into oocytes of *Xenopus laevis* as described elsewhere (14). The injected oocytes were incubated individually in microwells (pretreated with BSA) in 10 ul Barth's incubation medium (14), plus gentamycin. After 48h, the supernatants from live oocytes were pooled, made 25 ug/ml in PMSF and assayed for either IL2 or CSF activities.

RESULTS

When injected with poly A⁺ RNA isolated from PMA stimulated EL4 cells, *Xenopus* oocytes synthesize and secrete IL2 (9). Based upon a series of time course experiments (RCB unpublished), poly A⁺ RNA was isolated from EL4 cells stimulated for 12–14h rather than 24–48h as previously reported. As expected for a secreted protein, IL2 synthesized in oocytes was secreted into the supernatant. However we have observed that when very active mRNA is injected, substantial amounts of activity are also found inside the oocyte (Table 1A). Both supernatants and homogenates are somewhat inhibitory in the IL2 assay.

We were not able to obtain any colonies when homogenates of oocytes that had been

TABLE 1. Assay for IL2 and GM-CSF activities produced by microinjected *Xenopus* oocytes.

RNA	A. IL2 ASSAY ¹²⁵ IUdR incorporated, cpm.		B. GM-CSF ASSAY	
	Oocyte	Supernatant	Colonies	Clusters
Water Injected	80±10	80±20	4±2	6
Poly A ⁺ Injected	320±150	800±200	8±2	16±3
Gradient Injected	3050±375	2550±350	40±7	23±4
Maximum Response	9000±1500		--	
Control	400±270		2±1	6±1

Samples of RNA, both poly A⁺ and gradient purified, were dissolved in water at 1 mg/ml and 30 nl was injected into each oocyte. After 48h incubation live oocytes were pooled and assayed. A, IL2 bioassay: Incorporation of ¹²⁵IUdR into the CTL line MTL 2.8.1. 0.5 oocyte equivalent of both homogenates and supernatants were assayed in triplicate. Maximum stimulation measures incorporation of label at saturating levels of IL2, in the negative control no IL2 was added. B, GM-CSF bioassay: 1 oocyte supernatant equivalent was added to nucleated bone marrow cells suspended in medium as described in Materials and Methods. Samples (in triplicate) were incubated at 37°. Colonies (>50 cells) and clusters (<50 cells) were scored after 7 days. No exogenous factors were added in the negative control.

FIGURE 1

Sedimentation of IL2 and GM-CSF mRNAs on a sucrose gradient. Poly A⁺ RNA from PMA stimulated EL4 cells was sedimented through a 10–28% sucrose gradient, with markers of 4S tRNA and 28S and 18S rRNAs, at 48,000 rpm for 5h at 4°. The fractions were precipitated, dissolved in water and then injected into oocytes. The supernatants of oocytes were assayed for IL2 (○—○) and GM-CSF (●—●) activities as described in Materials and Methods.

injected with mRNA were used to stimulate mouse bone marrow cultures. Lusic *et al.* (15), who recently reported the successful translation of mRNA for human GM-CSF, encountered a similar difficulty. They attributed this phenomenon to an inhibitor of colony formation not observed when supernatants of mRNA injected oocytes were used, instead of the oocyte homogenates. Therefore, for subsequent experiments, we did not break up the oocytes, but performed the colony assay using supernatants of mRNA injected oocytes.

Using supernatants of water injected oocytes to stimulate mouse marrow cultures (1 oocyte equivalent per plate), we observed the presence of 3 colonies and 6 clusters per plate (Table 1B). Approximately the same "background" was observed in plates that did not receive any exogenous stimulus. Addition of supernatants from oocytes injected with unfractionated mRNA (1mg/ml) resulted in development of 8 colonies and 16 clusters. Thus, we were able to demonstrate an increased GM-CSF in mRNA injected oocytes as compared to water injected oocytes, but the signal was low. The poly A⁺ RNA was therefore centrifuged through a sucrose gradient with several RNA markers. The RNA in individual fractions was precipitated with ethanol, resuspended in water at 1mg/ml and injected into oocytes. Supernatants of oocytes were used to stimulate bone marrow cultures. A profile of mRNA activity in the gradient is shown in Fig. 1. CSF activity was observed in the translated product of mRNA with a maximum at fraction 13, corresponding to a sedimentation coefficient of 8S. Oocyte supernatants were also analyzed for IL2. As shown in Fig. 1, the fractionation resulted in a clear separation of IL2 mRNA from GM-CSF mRNA.

FIGURE 2. Morphology of colonies stimulated by GM-CSF translated in frog oocytes. Colonies were photographed *in situ* through an inverted microscope. Three distinct morphologies were observed; (a) compact (b) tight centre with loose surrounds (c) loosely disperse.

IL2 mRNA peaked in fraction 11, corresponding to a sedimentation coefficient of 11.5S (9).

The sucrose gradient fractionation resulted in a considerable enrichment of mRNA for GM-CSF. In contrast to the few small colonies observed with unfractionated mRNA, the peak gradient fractions stimulated development of many large colonies containing up to 500 cells as well as an increased number of small clusters. Material from the peak fraction, corresponding to 30 ng of injected mRNA, stimulated the development of 40 large colonies.

The gross morphology of colonies induced by supernatants of oocytes, injected with

FIGURE 3. Morphology of cells stimulated by GM-CSF translated in frog oocytes. Cytosmears, prepared from picked colonies, were stained with Wright-Giemsa stain. The bar corresponds to 50 microns at both magnifications.

fractionated mRNA, is shown in Figure 2. Three types of colony were observed, (a) compact, (b) tight centre with loose surrounds, (c) loosely dispersed. Morphological examination of the colonies in situ, under x125 magnification revealed 70% contained predominantly macrophages, 20% granulocytes and 10% to be mixed granulocyte-macrophage. Colonies were picked, the cells were centrifuged (Shandon Southern Cytospin, 5 min., 500rpm) and stained with Wright-Giemsa stain. Figure 3 shows two magnifications of cells prepared in this way. Two morphologically distinct cells can be observed. The majority of cells are large mononuclear macrophages containing many cytoplasmic vacuoles. At the higher magnification, the smaller granulocytic cells, with their characteristic "horseshoe" nuclei, can be clearly seen.

DISCUSSION

In this paper we report the translation of mouse T lymphoma mRNA into biologically active GM-CSF in Xenopus oocytes. We have also achieved a separation of GM-CSF mRNA from mRNA coding for IL2 upon fractionation of poly A⁺ RNA according to size, by sucrose gradient centrifugation (9). Mouse T cell derived GM-CSF is encoded by an mRNA which is poly A⁺ and has a sedimentation coefficient of 8S. This corresponds to an mRNA of about 900 nucleotides and thus has more than enough coding capacity for a 23,000 MW glycoprotein such as GM-CSF (4). Determination of the sedimentation coefficient for an RNA molecule, based upon comparison with RNA standards, and the extrapolation to a coding capacity is very approximate. However, the absolute value of the sedimentation coefficient for GM-CSF mRNA is less important than the demonstration of its separation from IL2 mRNA.

Both clusters (<50 cells) and colonies (>50 cells) were observed when bone marrow cultures were stimulated with supernatants from oocytes, injected with GM-CSF mRNA. Stimulation of the most mature precursor cells results in clusters while colonies arise from less mature progenitors. The variation in colony size is due to stimulation of committed precursors of varying degrees of maturity (1). In addition to size heterogeneity, three types of colony morphology were observed. Compact spherical colonies, colonies with a tight centre surrounded by dispersed cells and, finally, loosely dispersed aggregate colonies were all seen in the same culture. The state of development of the component cells in a colony govern its shape. More mature cells tend to be more mobile than immature cells and thus are able to migrate in the surrounding semi-solid culture medium (1). In situ examination of colonies produced in the presence of oocyte supernatant revealed that macrophages predominated. This was confirmed when cytosmears were prepared and stained. We have partially purified GM-CSF from PMA

stimulated EL4 cells and find that it stimulates macrophage, granulocyte and mixed colonies. At low concentrations macrophage colonies developed, while at higher concentrations more granulocyte and mixed colonies were observed (H.H. unpublished). In summary, the activity synthesized by oocytes induces the growth of clones which correspond in cell types, size and shape to those stimulated by low concentrations of GM-CSF from PMA treated EL4 cells.

The induction of macrophage and granulocyte differentiation by GM-CSF has led to the suggestion of its use in restoring populations of these cells after cytotoxic therapy (16). In addition, experiments have shown that GM-CSF can induce the differentiation of some leukemic cells, *in vitro* and *in vivo*, to normal macrophages and granulocytes (17, 18), thus suggesting a novel approach for treatment of myeloid leukemia. Recently Nicola and Metcalf reported that this latter differentiation activity was separable from GM-CSF (19). There was, however, always some residual CSF activity associated with differentiation activity. This problem of assigning activities to factors is a common one in lymphokine research, and can only be resolved by a combination of further separation procedures and molecular cloning. The successful translation of mRNA for murine and human (15) GM-CSF opens up the way for cloning their complementary DNAs, thus enabling studies on regulation of expression, structure, function and potential efficacy as a therapeutic agent.

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