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MULTI-LEVEL REGULATION OF LYSOSOMAL GENE EXPRESSION IN LYMPHOCYTES

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The expression of the gene coding for the lysosomal enzyme, β-glucuronidase (Gus), was examined in functionally distinct T, B and plasma cell lines. Each of the different groups of cells had different intracellular levels of active Gus enzyme and numbers of Gus mRNA copies per cell. Analysis of the molecular forms of Gus mRNA and protein by Northern and Western blotting revealed that the different types of cells all produced a single mature 2.7 kb transcript and a 73 kDa polypeptide. However, the utilisation of the Gus mRNA to produce the Gus antigen, and the subsequent post-translational processing of the polypeptide to generate the mature, enzymically active Gus, were found to be cell type-specific. Control of the functional expression of the Gus gene is thus exerted at both the transcriptional and translational levels, and appears to differ between different types of lymphocyte.

Control of protein expression in many types of cells is exerted by regulatory mechanisms which primarily influence gene transcription (1, 2, 3, 4, 5). This is a particularly prominent feature of the differential expression of specialized genes such as those for globin (6), metallothionein (7) and the T cell receptor (8), which are developmentally regulated in a tissue-specific manner. In contrast to these processes which control the initiation of transcription and thus the level of mRNA available for translation, the regulation of 'housekeeping' genes which comprise more than 90% of the active genes in most cells (such as glyceraldehyde 3-phosphate dehydrogenase, dihydrofolate reductase and histone H4) appears to involve mRNA maturation, stability and possibly localisation (9, 10). In addition, the regulation of gene expression can also be achieved at the post-translational level, such as occurs for collagen type 1(11), the complement protein C2 (12), interleukin 1 (13), membrane immunoglobulin (14) and the low density

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lipoprotein receptor (15). Although the precise mechanisms which govern the utilization of RNA transcripts for protein synthesis and maturation are complex and still poorly understood, such studies have shown that the number of polypeptides produced per mRNA copy and the rate of formation of mature, functional protein per polypeptide translated are two major, distinct processes which comprise 'Translational Yield' (16).

Several recent reports have suggested that Translational Yield may determine tissue-specific differences in the activities of certain degradative enzymes (16, 17, 18). Such enzymes are generally considered to effect housekeeping functions by maintaining the balance between synthesis and degradation of endogenous macromolecules and the turnover of extracellular components. In lymphocytes they also perform specialized immune functions which are characteristic of the particular subset of cell. These include cytotoxic reactions directed against target cells (19, 20, 21), antigen processing for presentation (22, 23) and intracellular turnover of cytokines, adhesion molecules and other cell surface receptors (24, 25, 26, 27). Moreover, major changes in the lysosomal/endocytic compartment have been found to be associated with the activation of resting lymphocytes (28). Thus, the control of these enzymes is likely to play a fundamental part in the diverse functional repertoire of different sub-populations of lymphocytes. In the present study we show that there are immune cell-specific differences in both transcription and translation which control the activity of the lysosomal enzyme, β-glucuronidase (Gus).

METHODS

CELLS AND CELL CULTURE The following murine cell lines were used: BW 5147, a spontaneous AKR T-cell lymphoma ; DO-11.10, a Balb/c x AKR functional T-helper cell hybridoma ; K_2S, a C57/Bl/6 radiation-induced leukaemia virus (Rad-LV/Nu1) lymphoma with suppressor/cytotoxic T-cell function ; A20 and M12, spontaneous Balb/c B-cell lymphomas ; TA3, an M12 X LPS-stimulated Balb/c X AJ spleen cell hybridoma ; A3.4, a CBA/Ca X NS1 hybridoma ; HB32, an NSI X Ig-immunized Balb/c spleen hybridoma ; NS1, a Balb/c plasmacytoma (29). All cells were cultured at 37°C in RPMI 1640 medium supplemented with 10% heat inactivated foetal calf serum (FCS), 100U per ml penicillin, 100μg per ml streptomycin, 50μM 2-mercaptoethanol and 20mM Hepes, pH 7.4, in an atmosphere of 5% $C0_2$ in air. Cell numbers were determined by direct counting in a heamocytometer after staining with crystal violet in 0.1% acetic acid.

MEASUREMENT OF B–GLUCURONIDASE (GUS) ENZYME ACTIVITY Cell suspensions were washed free of medium with phosphate-buffered saline (PBS) and centrifuged and resuspended at a density of 1×10^7 cells per ml. Lysates were prepared using Nonidet NP-40 and assayed for Gus activity using a fluorimetric assay, as previously described (30). One unit (U) of enzyme activity is defined as equivalent to the hydrolysis of 1 nmol of substrate per h at 37°C. Specific activity is the number of Units of Gus activity per 10^6 cells used for the enzyme assay, which is the same as the number of μU per cell.

MEASUREMENT OF TOTAL CELLULAR RNA Suspensions containing 10^7 cells were washed with cold PBS, followed by absolute ethanol, ethanol:ether (3:1 v/v) and finally 0.2N perchloric acid (PCA) at 4°C. Pellets were extracted three times using 0.5 ml of 0.5N PCA for 20 min at 70°C, followed by centrifugation at 800 x g for 20 min. The combined supernatants were assayed for total cellular RNA using the orcinol method , and the values used to calculate the amount of total RNA per 10^6 cells.

MEASUREMENT OF GUS-SPECIFIC mRNA AND NORTHERN BLOTTING Total RNA was isolated from each of the lymphoid cell lines using the guanidinium isothiocynate/cesium chloride method (31, 32) . Gus mRNA levels in the preparations of total lymphoid cell RNAs were determined using a quantitative dot blot assay, as previously described (33, 34). Briefly, 2.0 µl aliquots of the RNA samples and of known amounts of a standard preparation of Gus mRNA (120 µg per gram of total RNA) were spotted onto nitrocellulose filters, air-dried and baked in a vacuum oven at 80°C for 2 h. The filters were then hybridized with a 1.0 Kb cRNA riboprobe produced using T3 RNA polymerase and uniformly labelled with [^{32}P]-UTP ($2x10^9$ cpm per µg; 5×10^7 cpm per ml), as described previously (34). The hybridization was carried out in FDS buffer (50% formamide, 5 x standard saline-citrate (SSC), 10 x Denhartds, 10% dextran sulphate, 0.5% SDS and 200 µg/ml denatured salmon sperm DNA), for 24h at 60°C, after which they were washed four times in 0.1 x SSC containing 0.1% SDS at 70°C and twice at room temperature with 3mM Tris-HCl, pH 9.0, and blotted dry. Autoradiography was performed by exposure to XAR-5 film. The spots were cut out and the amount of ^{32}P probe which hybridised specifically was measured. The amount of specific Gus mRNA in the total cellular RNA was calculated by comparison with the hybridization of the probe to known amounts of Gus mRNA used as standard. The number of transcipts per cell was calculated using 8.6×10^5 as the molecular weight of Gus mRNA (16).

For Northern blotting, between 5 and 25 µg of each RNA preparation was denatured at 55°C for 15 min and fractionated on 1% agarose/formaldehyde gels. After vacuum transfer and cross-linking to nylon fibres (Hybond-N+, Amersham U.K.), the filters were blocked for non-specific binding, hybridised with the radiolabelled riboprobe, washed and visualized as described above for dot blot analysis.

SDS-PAGE AND WESTERN BLOTTING Aliquots containing between 1 and 5×10^7 cells were centrifuged, the pellets washed three times with Tris-buffered saline (TBS) and then directly solubilized in electrophoresis sample buffer, as previously described. Samples were subjected to electrophoresis on 5-15% SDS-polyacrylamide gradient gels (SDS-PAGE) which were then electroblotted onto nitrocellulose filters (28). The membranes were blocked with 2% casein in PBS and the Gus antigen detected using a rabbit polyclonal anti-mouse Gus antibody followed by horseradish peroxidase-conjugated anti-rabbit IgG. Filters were washed with TBS containing 0.5% Tween-20 and developed until visible bands appeared (35).

QUANTITATION OF GUS ANTIGEN BY FLOW CYTOMETRY (FACS) Total cellular Gus antigen was measured using FACS as previously described (36). Briefly, cells were washed, fixed with paraformaldehyde (1.0% in PBS), permeabilised with saponin (0.1%) and incubated with the anti-Gus antibody followed by FITC-conjugated goat anti-rabbit IgG. After washing, the labelled cells were resuspended in PBS containing 2% FCS and the fluorescence intensities of 10,000 individual cells

measured using a FACScan Flow Cytometer (Becton-Dickinson, Oxford UK). The average fluorescence intensities (AFI) accurately reflect the actual molecular amounts of the Gus antigen, and were used to calculate the number of Gus polypeptides per cell as previously described (36).

RESULTS

B, T and plasma cell lines of known immunological lineage and function were all found to produce substantial levels of Gus activity. As shown in Table 1, although the enzyme was not restricted to any particular type of lymphoid cell , there was nevertheless a marked variation in the level of Gus activity between the different cell groups. The lowest enzyme levels were found among the T cell lines, which expressed between 4.5 and 6.1 μU per cell and which showed no significant difference between subsets with helper or cytotoxic/suppressor function (DO-11.10 and K_2S, respectively). In contrast, the B cell lines contained nearly three times the activity of the T cells (an average of 14.4 compared with 5.1 μU per cell, respectively). However, the plasma cell lines studied had by far the highest levels of Gus, between 81 and 169 μU per cell (NS1 and HB32, respectively), representing an average of 25 and 10 times the activities present in the T and B cell lines, respectively. All the lymphoid cell lines had very similar secretion rates, less than 5% of the total intracellular Gus activity being released into the supernatant when 10^6 cells were cultured in 1 ml of fresh medium for 24h (data not shown).

Quantitative dot blot analysis was carried out to measure the precise amount of Gus-specific mRNA produced by each of the cells, in order to determine whether

TABLE 1: GUS ACTIVITY AND mRNA IN LYMPHOID CELL LINES

Cell line	Gus Activity (μU per cell)	Gus mRNA (transcripts per cell)	Translational Yield* (μU per mRNA)
T cells			
BW5147	4.5	---	---
Do-11.10	6.0	37	0.162
K2S	6.1	44	0.139
	ave 5.5		
B cells			
A20	17.1	18	0.949
M12	14.6	20	0.730
TA3	14.5	31	0.468
	ave 14.4		
Plasma cells			
A3.4	156	93	1.676
HB32	169	54	3.129
NS1	81	---	---
	ave 135		

* The Translational Yield is calculated as the number of μU of Gus enzyme activity per cell produced per Gus mRNA transcript per cell.

the differences in enzyme activity were related to differences in the cellular levels of the Gus gene transcripts. As shown in Table 1, the steady-state number of Gus mRNA copies per cell also varied over a wide range, the plasma cells being the most active at the transcriptional level. Such cells had the greatest number of Gus transcripts (93 and 54 per A3.4 and HB32 cell, respectively), significantly higher than the B cell lines. Surprisingly, these latter cells had a lower number of Gus mRNA copies than did the T cell lines. Despite the production of less Gus transcripts by the B cells, however, they nevertheless more effectively utilized the Gus message for the synthesis of functionally active, mature enzyme. Thus, calculation of the number of Units of Gus activity produced per copy of Gus mRNA, i.e., the 'Translational Yield' (16, 17, 18), revealed that the B cells had a higher overall efficiency, compared with the T cells, of the various processes involved in converting the Gus message into enzymically active tetrameric Gus. The results in Table 1 also show that the plasma cell lines, which transcribed the greatest amount of Gus mRNA, also had the highest Translational Yield, having post-translational processes which produced far more active enzyme per transcript than either the B or T cells.

Differences in the utilization of the Gus message because of possible differences between the lymphoid cells in alternate mRNA splicing and/or maturation were investigated by examining the molecular size(s) of the Gus transcript(s). The results of Northern blotting analysis showed, however, that all the cell lines produced a single mature Gus transcript of 2.7 kb (Fig 1). There was no evidence of multiple Gus mRNAs in any of the lymphoid cells. The absence of multiple species of mRNA is also consistent with the additional finding, by Western blotting, that the Gus enzyme in all the cell lines was composed of a single polypeptide sub-unit of Mr 73,000, as shown in Figure 2. In these experiments there was no evidence of proteolytic peptide cleavage or the presence of Gus degradation products.

The above data also suggest that, in addition to the control of Gus gene transcription, differences in non-proteolytic, post-transciptional mechanisms are likely to play an important part in determining the characteristic levels of Gus activity in each of the groups of lymphoid cell. Translational Yield has been defined as the product of two principal components: first, the rate of production of nascent polypeptides from the Gus message and second, the post-translational processing events that generate mature, active tetrameric lysosomal enzyme from the polypeptides (16). To investigate these distinct aspects we assessed the relative amounts of Gus polypeptides produced in each of the lymphoid cell lines by using a polyclonal antibody to measure the total antigen by flow cytometry (FACS) after permeabilizing the cells with detergent, as described in the Material & Methods. The results in Table 2 show that the B cell lines produced an average of twice the number of Gus polypeptides compared with the T cells (AFIs of 672 and 372; 63.7

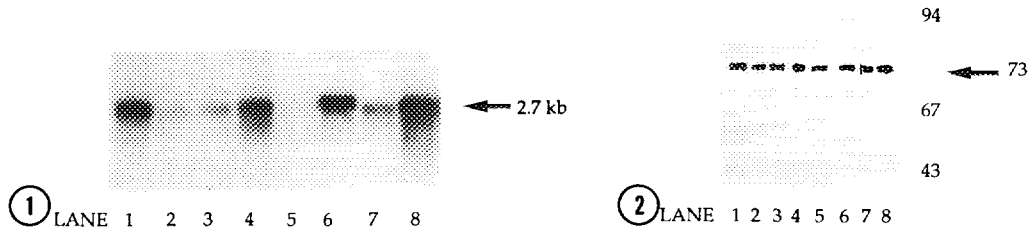

FIGURE 1.
ANALYSIS OF GUS mRNA BY NORTHERN BLOTTING
Northern Blot of lymphoid cell line Gus mRNAs, using between 5-25 µg of total RNA per lane. Lane: 1, D011-10; 2, K_2S; 3, A20; 4, M12; 5, TA3; 6, A3.4; 7, HB32; 8, NS1. Only one Gus mRNA transcript corresponding to a molecular size of 2.7 kb was present in the RNA extracted from the lymphoid cell lines.

FIGURE 2.
ANALYSIS OF GUS PROTEIN BY WESTERN BLOTTING
Western Blot of extracts of the lymphoid cell lines, using approximately 200U of Gus activity per lane. Lane: 1, BW5147; 2, D011-10; 3, K_2S; 4, M12; 5, TA3; 6, A3.4; 7, HB32; 8, NS1. The numbers on the right indicate the migration of standard proteins used as molecular weight markers. All the lymphoid cell lines show a uniform Gus polypeptide of Mr 73,000.

and 35.3 $x10^{-4}$ polypeptides per cell, respectively), while polypeptide levels in the plasma cells (214.5 $x10^{-4}$) were an average of 3 and 6 times higher than those of the B and T cells. However, calculation of the efficiency of mRNA utilization, i.e. of the relative amounts of polypeptides produced per Gus transcript, showed that the B cells and plasma cells were very similar, although both were much higher than

TABLE 2: GUS ANTIGEN AND TRANSLATIONAL YIELD

Cell line	Gus antigen per cell (AFI)	Gus polypeptides per cell ($x 10^{-4}$) +	Gus polypeptides per Gus transcript	Gus activity per Gus polypeptide (pU)	Translational Yield* (µU per mRNA)
Tcells					
BW5147	354	33.6	---	13.4	---
DO-11.10	347	32.9	8892	18.2	0.162
K2S	416	39.4	8955	15.5	0.139
	ave 372	ave 35.3		ave 15.7	
B cells					
A20	715	67.8	37667	25.2	
M12	526	49.9	24950	29.3	0.949
TA3	774	73.4	23677	19.8	0. 730
	ave 672	ave 63.7		ave 24.8	0. 468
Plasma cells					
A3.4	3306	313.4	33699	49.7	1.676
HB32	1853	175.7	32537	96.2	3.129
NS1	1630	154.5	---	52.4	---
	ave 2263	ave 214.5		ave 66.1	

* The Translational Yield is calculated as the product of the number of Gus polypeptides produced per Gus transcript and the number of units of Gus activity produced per Gus polypeptide.
\+ Calculated from the AFI, using the previously determined value that one AFI is equivalent to 237 molecules of the mature tetrameric Gus enzyme (36).

the T cell lines. In contrast, while the production of active enzyme from the antigenically reactive Gus peptides was similar in the T and B cells (15.7 and 24.8 μU), these post-translational processes were far more effective in the plasma cells (66.1 μU per polypeptide). The calculated 'Translational Yield' of Gus, i.e., the amount of active Gus enzyme produced per Gus mRNA transcript, is the same as that shown in Table 1, and again emphasises the marked differences in Gus regulation between the groups of lymphoid cells.

DISCUSSION

Enzyme, immunoassay and protein and RNA blotting have been used to examine the regulation of the gene for the lymphocyte lysosomal enzyme β-glucuronidase. We have found that the steady-state levels of enzyme activity differ among different lymphocyte cell types. To generate these differences, Gus gene expression appears to be regulated at three levels. First, the amount of Gus transcript varies among the three cell groups tested, suggesting that transcription and/or mRNA turnover rates are differentially regulated. Second, the efficiency with which Gus mRNA is translated into polypeptide recognised by antibody is significantly lower for T cells than for B cells or plasma cells. And third, the processing of the Gus polypeptides to the mature Gus enzyme yields a higher catalytic activity per molecule in the plasma cells than in the other two types of lymphoid cells.

A number of regulatory processes have previously been described which are involved in determining gene expression. The presence of multiple mRNA species, although coding for similar proteins, may differ in their efficiency of translation and thereby lead to significant differences in relative levels of protein expression and function. Alternate splicing of the same mRNA in different cell types might also account for wide variations in translational efficiency. In the present study, however, all the lymphoid cells examined contained a single Gus mRNA transcript, similar in size to the message found in a number of other types of cell and tissue (16, 18, 37). Although our experiments provide no evidence for the presence of any Gus transcript other than the 2.7 kb mRNA species, differences of 100 bases or less would not have been detected by Northern blotting. Such differences, for example in the 3' untranslated poly (A) region, could play an important part in regulating mRNA stability (9) as well as efficiency of translation (38). Differences in lymphoid cell Gus activities were also not due to proteolytic protein processing, since all the lymphoid cell lines produced a single polypeptide sub-unit and there was no evidence for the presence of cleavage products. These data suggests that lymphocyte-specific differences in Gus activity are likely to be exerted at the post-translational level as well as the transcription of the Gus gene.

'Translational Yield,' previously defined as the rate at which Gus mRNA transcripts are translated and the efficiency with which the nascent polypeptide chains are converted into enzymically active tetramers, has been shown to account

for specific difference in Gus synthesis and expression in a number of mammalian tissues (16, 17). Differential post-translational processing of the Gus enzyme has been found to occur within these different tissues, conferring differences in intracellular localization, secretion and functional activity of the enzyme. Similarly, in the lymphoid cell lines, although differences in intracellular levels of Gus can be accounted for partly by the amount of the Gus message produced by each of the groups of lymphoid cells, we found that differences in Translational Yield are also responsible for immune cell-specific Gus enzyme activities. As with many other degradative enzymes, Gus is synthesised on membrane-bound polysomes, the nascent polypeptides then translocated into the lumen of the endoplasmic reticulum and subsequently transported via the Golgi and trans-Golgi network ultimately to lysosomes (39, 40). A number of modifications occur during this maturation process, including proteolytic cleavage, the addition of oligosaccharides followed by specific trimming reactions, phosphorylation and the addition of complex oligosaccharides which can undergo sialylation (41, 42, 43) . Such reactions are likely to confer distinct catalytic activities with respect to the breakdown of both natural and synthetic substrates, and may also influence the susceptibility of the enzyme to degradative turnover. The results of the present study suggest that this cascade of post-translational modifications are all potential points for regulating Gus enzyme activity and appear to be unique in different types of lymphocyte.

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