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A 6-fold difference in the half-life of immunoglobulin μ heavy chain mRNA in cell lines representing two stages of B cell differentiation

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ABSTRACT

When B cells differentiate into plasma cells, there is a large increase in the cellular content of mRNA coding for immunoglobulin. This increase cannot be fully accounted for by the increase in rate of transcription of the genes. We have investigated the possibility that the half-life of μ heavy chain mRNA increases during B cell differentiation, by measuring the rates of decay of the same endogenous μ gene in two cell lines representing the B cell and the plasma cell. Using a pulse-chase protocol, it was found that there was a significant increase in the half-life of μ mRNA between the B cell and the plasma cell, and no detectable difference in the average half-life of total poly(A)+ RNA in the two cell lines. The reduced rate of decay of μ mRNA in the more differentiated cell type is almost sufficient to account for the difference in steady state μ mRNA levels between the two cell lines.

INTRODUCTION

The secretion of large amounts of specific immunoglobulin into the bloodstream plays a crucial role in the immune response in mammals. This event is triggered when resting B cells encounter antigen and are stimulated to divide and differentiate into antibody-secreting plasma cells; it involves a large increase in the cellular content of mRNA coding for secreted immunoglobulin (1,2,3). It is of interest to know the mechanism by which this increase in immunoglobulin mRNA levels is brought about.

The regulation of specific mRNA expression in mammalian cells can occur at various stages of mRNA metabolism, including transcription, processing of the primary transcript, transport to the cytoplasm and rate of cytoplasmic degradation

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(4). Recent evidence indicates that the increase in abundance of immunoglobulin mRNA when B cells differentiate cannot be fully accounted for by changes in the rates of transcription of the genes, and therefore must involve posttranscriptional processes (5,6,7,8). Studies with transformed cell lines which represent different stages of B cell development have been most informative in this respect. For example, the myeloma cell line MPC11 (equivalent to the plasma cell stage) was found to contain 150 fold more cytoplasmic μ mRNA than the lymphoma line 70Z/3 (representing a pre B cell), but their nuclear μ RNA levels differred by only 6 fold (5). Transcriptional run-off experiments have shown that whereas there is a 10-100 fold difference in μ heavy chain mRNA levels between cell lines representing early and late developmental stages, the rates of transcription of the μ gene differ by only 2-5 fold in these cells (6,7,8). Further evidence that transcription is not the major level of regulation is provided by transfection experiments which show that the heavy chain enhancer is fully active in pre B cells as well as plasma cell lines, indicating that it is not likely to be involved in the increase in mRNA accumulation at later stages of development (8).

Post-transcriptional regulation of gene expression, and in particular control of RNA stability, is emerging as an important mechanism for regulating gene expression in a variety of systems (9-14). We have therefore investigated the role of RNA stability in the expression of immunoglobulin genes, by measuring the relative rate of decay of μ mRNA derived from the same endogenous μ gene in cell lines representing the resting B cell and the plasma cell. To do this, a pulse chase protocol has been used, coupled with a ribonuclease protection method to detect μ mRNA. The data reveal a clear increase in μ mRNA stability between the resting B cell and plasma cell stages, which makes a significant contribution to the increased accumulation of μ mRNA at the plasma cell stage.

MATERIALS AND METHODS

Cell lines and plasmids. WEHI231 (15) and MXW231 (16) cells were a kind gift from Dr. M.S. Neuberger (M.R.C. Laboratory of Molecular Biology, Cambridge). MXW231 was provided with permission from Dr. W.C. Raschke, Developmental Biology Laboratory, Salk Institute for Biological Studies, San Diego. Both cell lines were grown in DMEM supplemented with 10% heat-inactivated foetal-calf serum and 50µM 2-mercaptoethanol. J558L cells (17) were also a gift from Dr. M.S. Neuberger; they were grown in DMEM supplemented with 10% heat-inactivated foetal calf serum.

The plasmids containing the probe sequences downstream of the bacteriophage SP6 promoter were constructed as follows. For the µNP probe, a 1.4kb *Pst* I fragment from pNP1 (18), containing a cDNA encoding the µ chain with specificity for the hapten 4-hydroxy-3-nitrophenyl acetyl (NP), was subcloned into the *Pst* I site of pSP64 (Promega Biotech) in the antisense orientation. For the rRNA probe, a 944bp *Hinc* II fragment from pMEB3 (ref. (19); a gift from Dr. M.S. Neuberger), containing the first 358bp of the murine 28s rRNA sequence and 586bp of upstream transcribed spacer, was subcloned into the *Hinc* II site of pSP65 (Promega Biotech), also in the antisense orientation. These plasmids were linearised at the *Eco* RI and *Hind* III sites in the polylinker, respectively, for the generation of RNA probes using SP6 polymerase.

Pulse chase. Cells in exponential growth were incubated at 5×10^6 per ml in growth medium containing 200µCi/ml [^{3}H]uridine (New England Nuclear), for two hours. They were then washed and resuspended at 4×10^5 per ml in medium containing 5mM of each of uridine and cytidine, for the chase period. At each time point during the chase, 10^7 cells were harvested and total RNA extracted. 150µg of RNA was analysed by ribonuclease protection with the µNP probe, and 800ng was hybridised to the rRNA probe.

Isolation and analysis of RNA. Total cellular RNA was isolated as described by Cathala *et al* (20), and quantitated by optical

density measurement at 260 nm.

SP6 polymerase transcription reactions were carried out essentially as described by Green *et al* (21), except that no ^{32}P-ribonucleoside triphosphate was added, and the concentration of ATP was increased to 1.5mM. The reactions were incubated at 40°C for two hours then treated with 10 units deoxyribonuclease (ribonuclease free from Pharmacia). Probes were recovered by phenol and chloroform extraction and unincorporated nucleotides were removed by several ethanol precipitations. The amount of probe synthesised was quantitated by measurement of optical density at 260nm.

Labelled total cellular RNA and unlabelled SP6 probes were hybridised for 20 hours according to the method of Hay *et al* (22) and ribonuclease digestion carried out as described by Zinn *et al* (23). Protected RNA fragments were analysed by electrophoresis through 3.5% polyacrylamide/8M urea gels. To visualise the ^{3}H-labelled bands, the gels were rinsed to remove the urea and then treated with "Amplify" (Amersham International) according to the manufacturer's instructions. They were then dried down and exposed to preflashed X-ray film. For quantitation, the bands were cut from the gel and the radioactivity solubilised by treatment with 1ml "Protosol" (New England Nuclear) and 0.3ml water, in glass scintillation vials at 37°C overnight. Samples were counted after addition of 5ml of aqueous scintillant. The recovery of radioactivity using this procedure was approximately 20%.

UTP Pool analysis. The specific activity of the UTP pool during the chase was determined as described by Brock and Shapiro (24). UTP standards were used to determine that the quantitation of peak area by integration was linear over a range of UTP concentrations. The specific activity was expressed as dpm per unit peak area.

Decay of Poly(A)+ RNA. ^{3}H-labelled total RNA was isolated at suitable time points from a pulse-chase experiment and poly (A)+ RNA selected by oligo (dT) cellulose chromatography (25). The specific activity of labelled poly(A)+ RNA during the chase was determined by measurement of optical density at 260 nm of an aliquot and liquid scintillation counting. The

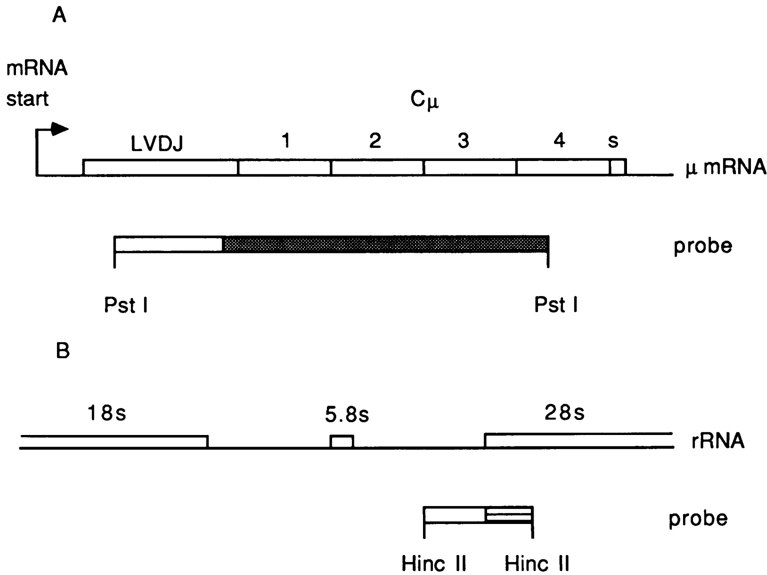

Figure 1. Probes for Northern blot analysis and Ribonuclease protection. (A) The μ mRNA is shown with the 1.4kb *Pst* I cDNA fragment from pNP1 (18) which was inserted into pSP64 for generation of RNA probes *in vitro*. The shaded area indicates the 1.1kb fragment which is protected when hybridised to μ mRNA from WEHI231 and MXW231 cells. (B) Part of the rRNA primary transcript is shown, with the 944bp *Hinc* II fragment from pMEB3 (19) which was inserted into pSP65 for generation of RNA probes *in vitro*. The shaded area indicates the 358bp fragment which is protected by hybridisation to 28s rRNA.

poly(A)+ RNA was analysed by Northern blotting with the rRNA probe, which showed that there was negligible contamination with ribosomal RNA (data not shown).

RESULTS

Cell lines.

The two murine cell lines chosen for study were WEHI231, a B cell lymphoma representing the resting B cell stage (15), and MXW231, a hybridoma formed by fusing WEHI231 with the IgG producing plasmacytoma MPC11 (16). The WEHI231 cells show characteristics of antigen sensitive B cells, their membranes contain monomeric IgM, and they do not secrete any detectable immunoglobulin, or make J chain (15,16). Like other

hybridomas, MXW231 shows characteristics of the plasma cell stage of differentiation (26-28). It continues to produce secreted IgG from the MPC11 parent, and in addition produces and secretes pentameric IgM; the μ heavy chain being derived from the WEHI231 gene (16). These cell lines provide the opportunity of studying the expression of the WEHI231 μ gene in two cellular environments, which mimic those of a B cell and a plasma cell. Northern blot analysis of poly(A)+ RNA confirmed that the two cell lines produced the expected species of μ mRNA (26,27,29) and showed that there is 10-15 fold more μ mRNA in MXW231 cells than in WEH1231 cells (data not shown).

Validation of the ribonuclease protection method.

In order to compare the rates of decay of μ mRNA in the two cell lines, pulse chase analysis was performed using [^{3}H]uridine, and a ribonuclease protection method was developed to detect specific labelled RNA. Total RNA isolated from cells labelled with [^{3}H]uridine was hybridised to an unlabelled antisense probe generated *in vitro* using SP6 polymerase (30) (see Materials and Methods). Unhybridised molecules were then removed by digestion with ribonuclease, and the remaining protected fragments analysed by polyacrylamide gel electrophoresis and fluorography. Quantitation was achieved by cutting the bands from the gel and subjecting them, after solubilisation, to liquid scintillation counting. This method has the advantage that any possible degradation products which hybridise to the probe can be distinguished by the gel electrophoresis step. The μNP and rRNA probes used in this analysis are shown in Fig.1. The μNP probe (Fig.1A.), protects 1.1kb of μ mRNA derived from the WEHI231 μ gene, and the rRNA probe (Fig.1B) protects 358bp of 28s rRNA.

Preliminary experiments were carried out to optimise ribonuclease digestion conditions with respect to signal to noise ratio. For hybridisations containing 150 μg labelled RNA and 1μg μNP probe, optimum conditions were found to be digestion at 18°C for 30 minutes with 10 μg/ml ribonuclease A and 0.5 μg/ml ribonuclease T1. Using these conditions, no

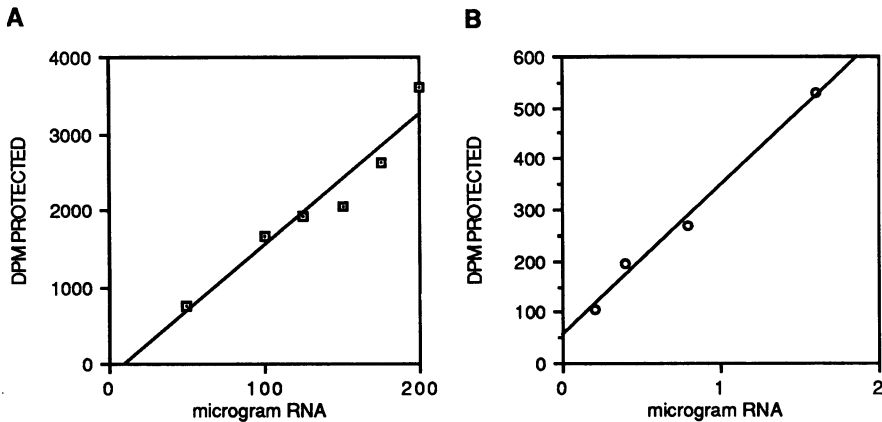

Figure 2. Validation of hybridisation method. MXW231 cells were labelled with [^{3}H]uridine for two hours as described in Materials and Methods for the pulse-chase experiments, and total RNA was prepared from the cells. Increasing amounts of this labelled RNA were then hybridised to a constant amount (1µg) of each of the probes, and ribonuclease protection carried out. After the protected fragments had been resolved by polyacrylamide gel electrophoresis, they were cut from the gel and the radioactivity quantitated by liquid scintillation counting; here the protected counts are plotted against RNA input. (A), µNP probe; (B), rRNA probe.

bands were observed in control hybridisations which did not contain probe (not shown). However, some background remained due to incomplete digestion of the labelled RNA, which appeared as a smear on the autoradiograph (for example, see Fig.3A and 3B). This could be removed by increasing the temperature of digestion or the amount of ribonuclease, but at the expense of some loss of signal caused by nicking by the ribonuclease at mismatches in the constant region between the µNP probe and the µ mRNA, which are of different allotypes (15,18,31). It was demonstrated, however, that the signal obtained by cutting out the 1.1kb band is linear over a range of RNA amounts hybridised. Increasing amounts of ^{3}H-labelled total RNA from MXW231 cells were hybridised to 1µg µNP probe, and the radioactivity in protected fragments was quantitated as described above (Fig.2A). A similar experiment for the rRNA probe was performed (Fig. 2B), and signals from both

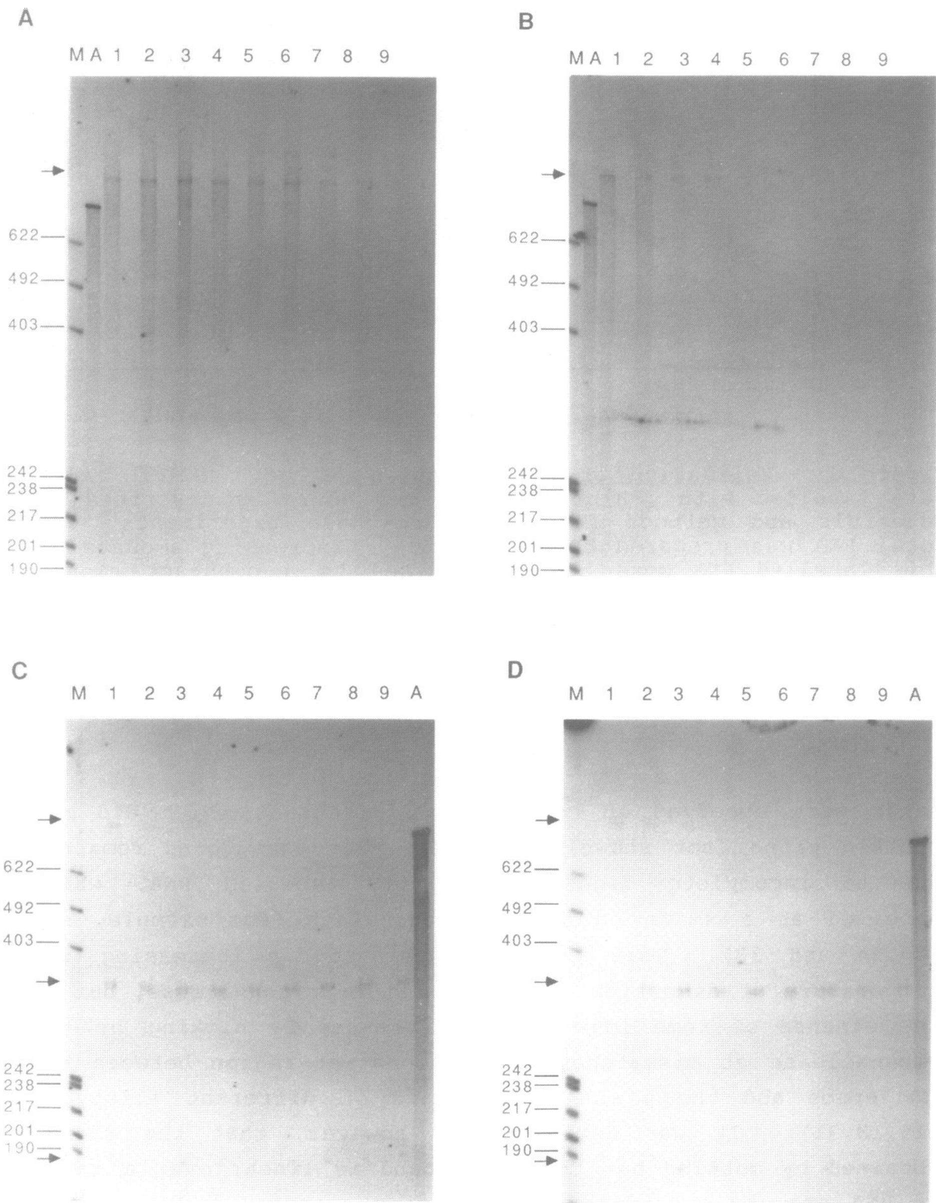

__Figure 3. Pulse chase and ribonuclease protection of WEHI231 and MXW231 RNA__. Pulse chase and ribonuclease protection were carried out as described in Materials and Methods. (A), MXW231/μNP probe; (B), WEHI231/μNP probe; (C), MXW231/rRNA probe; (D), WEHI231/rRNA probe. Lane M, end-labelled markers

probes can be seen to be linear over the range of RNA amounts used.

Pulse chase experiments.

Pulse chase analysis was carried out as described in Materials and Methods, and RNA isolated at suitable time points during the chase was hybridised to the μNP and rRNA probes as before. Fig.3 shows the ribonuclease protection for one such experiment. The μNP probe protects a band of 1.1kb, which clearly decays faster in WEHI231 cells than in MXW231 cells (Fig. 3A, 3B; arrow), whereas the 358 nucleotide rRNA band has a similar rate of decay in both cell types (Fig. 3C, 3D; middle arrow). The rRNA probe also protects a longer band of around 940 nucleotides, and a smaller band of approximately 200 nucleotides (Fig. 3C, 3D; top and bottom arrows respectively). It is possible that the longer band is produced by hybridisation to a rRNA precursor molecule; its rapid rate of decay is consistent with this interpretation. The smaller band decays at the same rate as the main band, and could be the result of secondary structure effects, or be produced by hybridisation to shorter probe molecules arising from early termination of the SP6 polymerase. Its origin was not investigated further. The rRNA is very stable in both cell lines, and the small reduction in intensity of this band which is observed is mainly due to the dilution effect of cell division; the actual half-life of rRNA in dividing cells is of the order of 100 hours, and it can be assumed to be essentially stable (10,32-38).

The radioactivity in the protected fragments was quantitated as before and the results of the two such experiments are shown in Fig.4, where the signal for μ mRNA has been normalised to that for rRNA. As was apparent from examination of the gels in Fig. 3, the μ mRNA is much longer-lived in MXW231 cells than in WEH1231 cells, measured

generated by Hpa II digestion of plasmid pAT153. Lane A; an 834 base SP6 RNA labelled with [^{3}H]GTP loaded as a control for the fluorography procedure and the recovery of radioactivity from gel slices. Time during chase was as follows: Lanes 1-9 represent 0, 2, 4.25, 10.5, 15.5, 20, 28.5, 38.25 and 44 hour time points respectively.

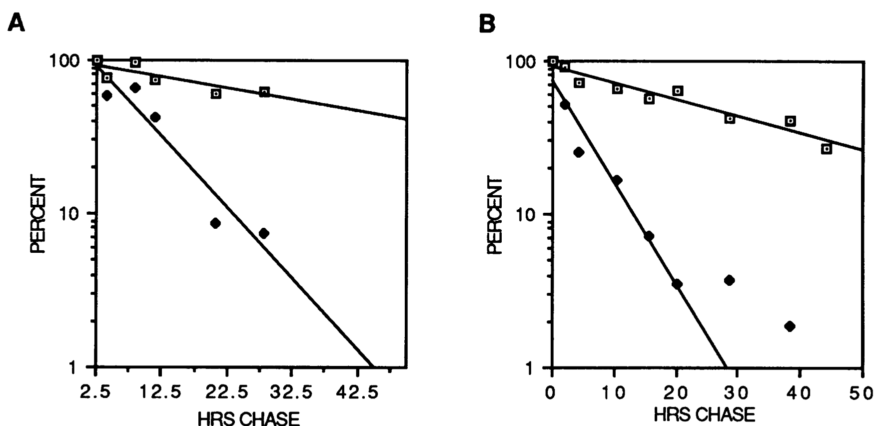

Figure 4. Quantitation of radioactivity in protected fragments from pulse chase experiments. The radioactivity contained in protected fragments from pulse chase experiments was measured by liquid scintillation counting as described in Materials and Methods. The data from two independent experiments are shown; (B) represents the data from the experiment shown in Figure 4. In each case, the protected counts from the μNP probe were normalised to those from the rRNA probe at each time point during the chase, and expressed as a percentage of the first time point. Initial dpm protected by the μNP probe were as follows: WEHI231, 3139 and 3090 (filled symbols); MXW231, 1475 and 2591 (open symbols). Linear regression analysis was used to determine the lines of best fit from which the half-lives were calculated.

relative to ribosomal RNA. The mean half-life of μ mRNA was found to be 5.5 hours in WEH1231 cells and 34 hours in MXW231 cells (Table 1), indicating approximately a 6 fold longer half-life in the more differentiated cell type. This difference in rates of decay would make a significant contribution to the difference in steady state μ mRNA levels observed between these cell lines, but does not fully account for it (see discussion).

Decay of UTP pool.

Measurement of the half-life of mRNA in mammalian cells by a pulse chase protocol is made difficult by the large size of the ribonucleotide pool and by the fact that the phosphorylated precursors cannot be chased out of the cells (10,39-41). Therefore it is important to determine whether there is any differential effect due to continued

Table 1. Half-life of μmRNA in WEHI231 and MXW231 cells. Results of the two experiments shown in Fig. 5. 95% confidence intervals are given in brackets.

	HALF-LIFE (hrs)		
	WEHI231	MXW231	RATIO
1	6.5 (4.7-10.5)	40.7 (>20.4)	6.2
2	4.6 (3.6-6.3)	27.7 (22.2-37.0)	6.1
mean	5.5	34.2	6.2

incorporation of label between the two cell lines, which could contribute to the observed difference in μ mRNA half-life. To obtain an estimate of the rate of decay of the UTP pool, its specific activity was measured during a pulse chase experiment using the method of Brock and Shapiro (24). The amount of UTP in cell lysates was quantitated by HPLC, using an anion exchange column and eluting the nucleotides with an ammonium phosphate gradient. The radioactivity in the UTP peak was determined by liquid scintillation counting. The results of this experiment are shown in Fig. 5A. It can be seen that the specific activity of the ribonucleotide pool does indeed remain high, as expected, for the initial part of the chase, but there is no significant difference in the chase conditions between the two cell lines. The high initial specific activity of the UTP pool will therefore introduce a greater error into the measurement of the shorter half-life molecules (see discussion).

Decay of total polyadenylated RNA.

To determine whether the difference in half-lives is specific for μ mRNA, or whether it applies to the general population of mRNA in the cells, a pulse chase experiment was carried out as normal and total polyadenylated RNA was isolated at various time points during the chase. The material which bound oligo(dT) cellulose was analysed by Northern blotting with an rRNA probe to demonstrate that it contained only negligible amounts of contaminating rRNA (not

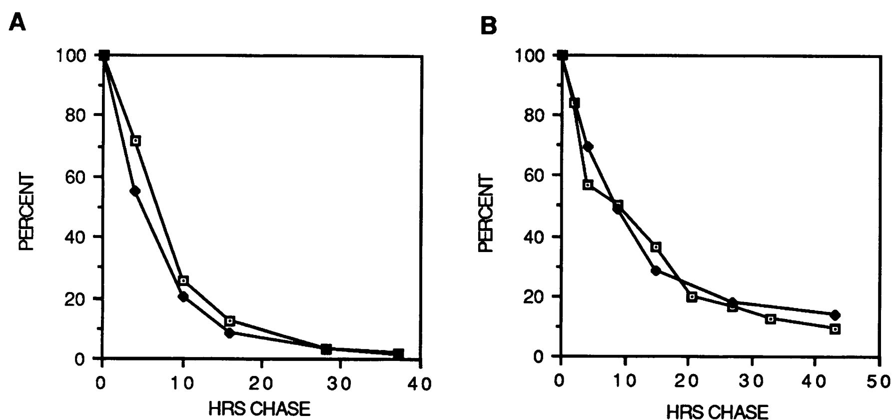

Figure 5. Decay of UTP pool and total poly(A)+ RNA. (A) The specific activity of the UTP precursor pool in WEHI231 and MXW231 cells was measured at several time points during a chase as described in Materials and Methods. It is plotted here as percent of maximum versus time during the chase. Maximum values expressed as dpm per unit peak area were 3590 for WEHI231 (filled symbols) and 1293 for MXW231 (open symbols).
(B) Polyadenylated RNA was prepared from WEHI231 and MXW231 cells at various time points during a chase, as described in Materials and Methods. Its specific activity was measured and expressed as a percentage of the maximum, then plotted against time during the chase. Maximum values were 3.88×10^5 dpm/µg for WEHI231 (filled symbols) and 1.95×10^5dpm/µg for MXW231 (open symbols).

shown). The specific activity of the poly(A)+ RNA is plotted as a function of time in Fig.5B. It can be seen that the decay of poly(A)+ RNA is very similar in the two cell lines; also the complex pattern of decay of poly(A)+ RNA is consistent with that seen by others (10,32,34). It is clear, therefore, that within the limits of detection of this method, there is no significant difference in the mode of decay of total poly(A)+ RNA between WEHI231 and MXW231 cells. Some additional control must therefore be exerted over the degradation of µ mRNA to account for the observed difference in half-lives.

DISCUSSION

The data presented in this paper show, using a pulse-chase protocol, that µ mRNA has approximately a 6 fold longer

half-life in the MXW231 hybridoma cell line than in the B cell lymphoma WEHI231, measured relative to ribosomal RNA. It is clear however that the use of a pulse-chase method with mammalian cell lines presents problems due to continuing incorporation of labelled nucleotides at the start of the chase. This is illustrated by the fact that the specific activity of the UTP pool takes approximately 15 hours to decay to 10% of its initial value (Fig.5A). In general this makes the method unreliable for accurately determining the decay rates of short-lived messages; so the WEHI231 half-life values in particular should be interpreted with caution. However, the chase conditions are very similar for the two cell lines (Fig.5A), and as the rates of transcription of the µ gene are also very similar (42), the observed difference in half-lives is unlikely to be an artifact of the chase protocol.

There is no detectable difference in the rate of decay of total poly(A)+ RNA, suggesting that some specific control must be exerted over the decay of µ mRNA. A 6-fold difference in half-life, together with the 1.5 fold difference in µ gene transcription observed by Mason _et al_ (42), almost fully accounts for the 10-15 fold difference in steady state levels of µ mRNA (not shown). The discrepancy could be due to inaccuracies in the measurements, or due to additional regulation at other levels of mRNA metabolism, for example mRNA processing or nuclear to cytoplasmic transport.

Others have also measured µ mRNA decay rates in immortalised cell lines representing different stages of B cell differentiation. Jack and Wabl (43) used transciptional inhibitors to prevent mRNA synthesis, and showed, (although they did not calculate half-lives), that µ mRNA was more stable in plasmacytomas and hybridomas than in B cell lines, consistent with our result. Mason _et al_ (42) used a heat shock promoter driving a µ gene to induce a pulse of µ mRNA synthesis, and demonstrated a half-life of 14 hours in MXW231 cells and 3 hours in WEHI231, a 4.5 fold difference. The quantitative difference between their results and ours is probably a reflection of the difficulty in making these measurements.

There are several possible reasons for the reduced rate of decay of μ mRNA in cells representing the plasma cell stage. It could be argued that it is an artifact caused by the use of immortalised cell lines. This is unlikely, however, as the relative transcriptional rates and steady state RNA levels agree qualitatively if not quantitatively with data from primary B cells (6,7,44). Our experiments do not distinguish between μ_s and μ_m RNA, and it is possible that the difference in decay rates is due to μ_s mRNA being intrinsically more stable than μ_m mRNA at both stages of differentiation, or due to selective stabilisation of μ_s mRNA at the plasma cell stage. These possibilities have not been formally tested, but several observations make them unlikely. Jack and Wabl's results show that there is no difference in the stabilities of μ_s and μ_m mRNA at the pre-B and B cell stage, both being unstable (43); therefore there is no intrinsic difference in their stability. Selective stabilisation of μ_s mRNA in plasma cells is also unlikely, because when constructs which only allow the production of μ_m have been transfected into myeloma and hybridoma cells in transient and stable expression assays, the resulting messages are expressed at levels comparable to those of μ_s (45-49). Therefore it is probable that the stabilisation mechanism does not distinguish between μ_s and μ_m.

Evidence is accumulating that the sequences that determine the stability of μ mRNA reside in the 5' region of the molecule. These sequences may interact directly or indirectly with ribonucleases, or may be important for controlling the rate of translation of the message, or its subcellular localisation. Kelley and Perry (7) showed that "sterile" transcripts, which initiate downstream of the V region promoter and do not contain the 5' leader, are not regulated in the same way as productive transcripts when B cells differentiate to plasma cells. In addition, Mason et al have suggested that the localisation of the message onto membrane-bound polysomes is important for its stabilisation (42). The subcellular localisation of histone mRNA has also been shown to be important for the correct regulation of its stability when DNA synthesis is inhibited (50). While this

manuscript was being prepared, Jack et al reported that nonsense mutations destabilise μ mRNA, presumably by decreasing ribosome loading (51). Further experiments are required, however, to determine whether differences in ribosome loading are responsible for the change in μ mRNA half-life when B cells differentiate.

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