The EMBO Journal vol.7 no.4 pp.1041 – 1046, 1988

Immunoglobulin mRNA stability varies during B lymphocyte differentiation

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Communicated by G.Köhler

During differentiation of B lymphocytes, the change in the amount of immunoglobulin heavy chain produced is reflected by a change in the steady state level of heavy chain mRNA. At the pre-B cell stage, the earliest stage at which immunoglobulin chain is produced, and later at the small resting B cell stage, there is a low steady state level of heavy chain mRNA. After the small B cell has differentiated to become a plasma cell, the steady state level of heavy chain mRNA is much higher. We confirm that the transcription rate at the immunoglobulin μ heavy chain gene does not change during differentiation from the pre-B cell to the plasma cell stage. In contrast, we show here that differences in the stability of μ mRNA are sufficient to account for the differences in the steady state level at the various differentiation stages.

Key words: rate of transcription of μ gene/steady state level of μ mRNA/inhibition of μ gene transcription

Introduction

Pre-B lymphocytes are the precursors of small B lymphocytes (Raff *et al.*, 1976). Pre-B cells produce cytoplasmic immunoglobulin heavy chain (Burrows *et al.*, 1979). When the cells start to synthesize light chain, they become B cells, which can be triggered by antigens or mitogens to differentiate into plasma cells. There is less heavy chain in the cytoplasm of pre-B cells and B cells than in the cytoplasm of plasma cells (Raff *et al.*, 1976; Burrows *et al.*, 1979), which secrete large amounts of the complete immunoglobulin molecule consisting of both heavy and light chains. The steady state level of heavy chain mRNA has been measured in lipopolysaccharide (LPS)-stimulated B lymphocytes and in cell lines derived from the pre-B, B and plasma cell stages. In LPS-stimulated B lymphocytes there was a 25- to 80-fold increase in μ heavy chain mRNA (Yuan and Tucker, 1984; Lamson and Koshland, 1984; Berger, 1986). Pre-B and B cell lines have 10–100 times less μ heavy chain mRNA than do plasmacytomas and hybridomas (Perry and Kelley, 1979; Eshhar *et al.*, 1979; Perry *et al.*, 1981; Nelson *et al.*, 1983; Wabl and Burrows, 1984). This variation in mRNA level could, in principle, result from different rates of transcription at the heavy chain locus, but apparently it does not. Although there is some variability in rate, perhaps due to differences in promoter strength among the variable region gene segments, it has been concluded from experiments with transformed cell lines that once a transcriptional unit is created, the transcription rate at the heavy chain gene locus remains the same in pre-B, B and plasma cells (Yuan and Tucker, 1984; Gerster *et al.*, 1986; Kelley and Perry, 1986). Here we confirm this conclusion by demonstrating that a given μ gene is transcribed at the same rate in both pre-B and plasma cells, but that the plasma cells have a higher steady state level of μ mRNA. Therefore, post-transcriptional events must account for the differences in μ mRNA levels at the various cell stages. A difference in mRNA stability is one factor that can lead to a difference in steady state levels. Here we compare the stability of μ mRNA in cell lines representing the pre-B, B and plasma cell stages.

Results and discussion

Steady state levels of μ mRNA in various cell lines

The cell lines used in this study, which represent the pre-B, B and plasma cell stages, are listed in Table I. FH is the product of a fusion between F, an Abelson virus-transformed pre-B cell line, and Ag8.653, a myeloma that has lost its functional immunoglobulin genes and has no gene segment for the constant region of the μ chain (Cμ). KH is a fusion product of K, another Abelson virus-transformed pre-B cell line (Weimann, 1976), and Ag8.653, and Sp7 is a fusion product of a spleen cell with the myeloma X63. All of the cell lines except Ag8.653 and A1.77 produce μ heavy chain. Hybridomas are polyploid and, therefore, often lose chromosomes. By cytoplasmic immunofluorescence with antiserum to μ chain, we determined the percentage of cells that actually produce μ chain. Indeed, we found that only 10% of the cells in line KH were μ positive. All of the other μ-producing lines had been recently subcloned, and >98% of the cells were μ positive.

The cell lines described here can be classified as having high or low steady state levels of μ chain mRNA, based on a dot blot titration of mRNA hybridizing to a μ cDNA probe (Figure 1). Plasmacytoma MOPC-104E and hybridomas

Table I. Characterization of cell lines used in this study

Cell line[a]	Cell type	Number of Cμ alleles	Reference[b]
A1.77	Pre-B cell	1	Wabl and Burrows, 1984
F (A33-11.88)	Pre-B cell	1	Wabl *et al.*, 1984
K (K.40.54)	Pre-B cell	2	Beck-Engeser *et al.*, 1987
230-37	Pre-B cell	2	M.Wabl, unpublished
70Z/3	Pre-B cell	2	Nelson *et al.*, 1983
38C-13	B cell	2	Nelson *et al.*, 1983
FH (H22-11-12)	Plasma cell	1	Wabl *et al.*, 1984
KH	Plasma cell	?	This study
Sp7	Plasma cell	?	Köhler and Milstein, 1976
MOPC-104E	Plasma cell	?	Potter, 1972
Ag8.653	Plasma cell	0	Wabl and Burrows, 1984

[a]Previous cell line designations are given in parentheses.
[b]The references do not necessarily describe the origin of a line, but rather its complement of Cμ alleles.

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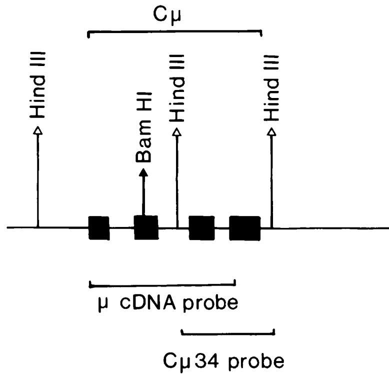

Fig. 1. DNA probes covering parts of the Cμ gene segment.

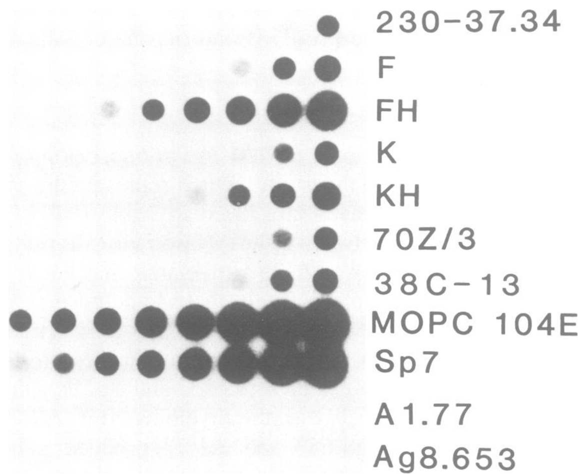

Fig. 2. Titration of steady state level of μ chain mRNA by dot blot hybridization. The denatured cytoplasm from 2×10^5 cells was serially diluted in 2-fold steps. Hybridization was performed with a μ cDNA probe (shown in Figure 1). Clone 230-37.34 is a subclone of line 230-37.

Sp7 and FH contain 8–100 times more μ mRNA than do the pre-B cells 230-37.34, F, K and 70Z/3, and the B cell 38C-13 (Figure 2). Since only 10% of the KH cells are μ positive, the titer should be multiplied by 10 for comparison, which places KH into the 'high' category as well. Lines A1.77 and Ag8.653 produce no μ RNA transcripts (H.-M.Jäck and M.Wabl, unpublished) and serve as controls for the hybridization specificity of the μ cDNA probe for μ RNA. Importantly, fusion of pre-B cell lines F and K to the myeloma Ag8.653 (producing hybridomas FH and KH, respectively) increases μ mRNA levels by factors of 8 and 20, respectively.

Rates of μ gene transcription

To compare transcription rates at the heavy chain locus in the cell lines in which we measured steady state levels of μ mRNA, we performed 'nuclear run-on' experiments (McKnight and Palmiter, 1979; Groudine *et al.*, 1981; Linial *et al.*, 1985). In this assay, radioactively labeled RNA is synthesized in isolated nuclei by RNA polymerases and hybridized to filters carrying the plasmid pBR322 with a genomic *Hin*dIII insert covering the third and fourth exons of Cμ (Cμ3 and Cμ4) or to filters carrying the single-stranded

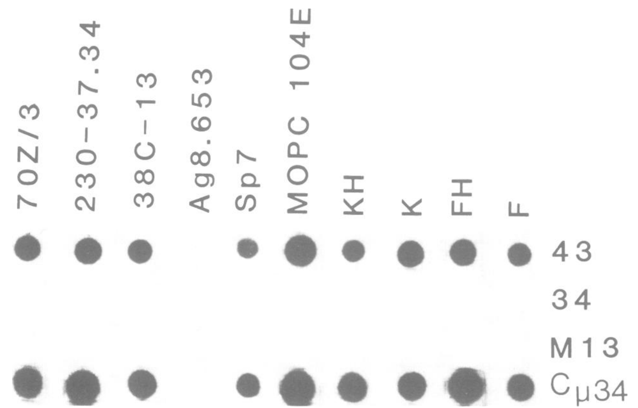

Fig. 3. Nuclear run-on experiment to compare transcription rates at the μ locus of various cell lines. **43**: M13μ43 probe, single-stranded M13 probe containing exons Cμ4 and Cμ3 in that orientation, and binding to sense transcripts. **34**: M13μ34, containing exons Cμ3 and Cμ4 and binding to antisense transcripts.

M13 DNA with the same insert (Figure 1).

For each set of measurements we verified that the hybridization signal was proportional to RNA input. In Figure 3, the transcription rates of the lines listed in Table I are compared. The transcription rate of the plasmacytoma MOPC-104E is rather higher than in the other cells. This is in good agreement with the fact that MOPC-104E also has about 2–4 times more μ mRNA than plasma cell hybridoma Sp7 (Figure 2). However, MOPC-104E has about 60 times more steady state μ mRNA than B cell line 38C-13. The μ gene in Sp7 is transcribed somewhat less than in clone F (Figure 3), although Sp7 has about 30 times more steady state μ mRNA (Figure 2). Clone KH transcribes only a little less mRNA than the other cell lines, even though only 10% of its cells are μ positive. We know that in the K cell line both μ alleles are transcribed (Beck-Engeser *et al.*, 1987) and, therefore, both contribute to the transcription rate. This is presumably also the case for KH. Lines F and FH contain only one μ gene, which is the same in both lines; they differ by a factor of 8–16 in their steady state level of μ mRNA (Figure 2), yet their transcription rates differ by a factor of no more than 2. All in all, these lines, whether of pre-B, B or plasma cell type, show a remarkably similar transcription rate at the μ locus. We found the same result with filters carrying double-stranded plasmid DNA and those carrying single-stranded M13 DNA in the Cμ4–Cμ3 (i.e. reading strand) orientation. No Cμ sequences hybridized to the M13 single-stranded DNA with the insert in a Cμ3–Cμ4 (i.e. nonreading) orientation or to M13 with no insert.

In summary, the transcription rates of pre-B, B and plasma cells do not differ systematically. The small difference found in MOPC-104E probably reflects the promoter strength, not the differentiation stage.

Stability of μ mRNA in various B-cell differentiation stages

Because transcription rates in these cell lines are so similar, post-transcriptional regulation must account for the different levels of steady state μ mRNA. One likely possibility is mRNA stability. By incubating cells with actinomycin C, which inhibits transcription in cell culture (Kersten and Kersten, 1974), we determined the amount of μ mRNA left

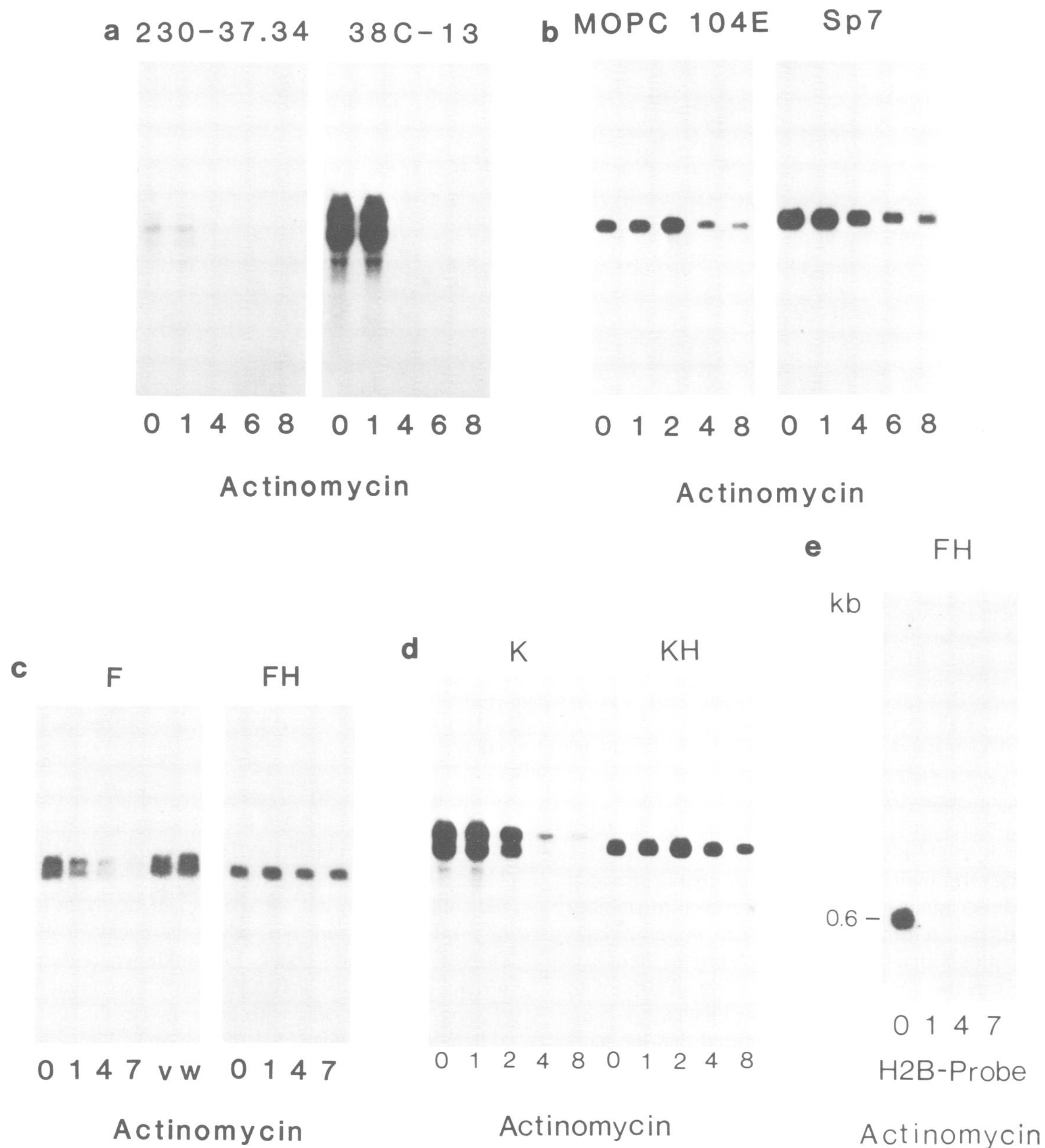

Fig. 4. Northern blot analysis of μ mRNA isolated from cells at various times after addition of actinomycin C to the culture. Filters with bound mRNA were probed with nick-translated μ cDNA. (**a**) Pre-B and B cells; (**b**) plasma cells, (**c**) pre-B cell line F and hybridoma FH; (**d**) pre-B cell line K and hybridoma KH; (**e**) blot for FH shown in (c) rehybridized with a histone 2b (H2B) probe. The numbers below the lanes indicate the number of hours after addition of actinomycin C. In (c) lane v: without actinomycin C; lane w, without actinomycin C but with dimethyl sulfoxide, the solvent for actinomycin C.

in the cells at various times. Reliable measurements of c-*myc* RNA turnover using actinomycin C have been reported for HeLa cells (Dani *et al.*, 1984) and for mouse plasmacytomas and an Abelson virus-transformed pre-B cell line (Piechaczyk *et al.*, 1985). Actinomycin C intercalates into DNA and thereby efficiently blocks RNA transcription. Ten minutes after addition of the drug, transcription is completely blocked in plasmacytomas (Piechaczyk *et al.*, 1985). To verify this effect in our cell lines, we first determined [^{3}H]-uridine incorporation in the cells after incubation with actinomycin C. One hour after addition of actinomycin C, [^{3}H]uridine was no longer incorporated into RNA in any of the lines tested (not shown).

The amount of μ mRNA left in the cells at various times after addition of actinomycin C was then determined by Northern blot analysis (Figure 4). In the pre-B cell line 230-37.34 and in the B cell line 38C-13, most of the μ mRNA had decayed after 4 h of incubation (Figure 4a). In sharp contrast, about half of the mRNA was still present after 4 h in plasma cell lines MOPC-104E and Sp7 (Figure 4b). We found an analogous result for the pairs of cell lines, F and FH, and K and KH. Thus, after 4 h of incubation with actinomycin C, the amount of μ mRNA remaining in pre-B cell lines F and K had clearly dropped, while in the hybridomas representing the plasma cell stage, FH and KH, there was almost no decay of μ mRNA after 7–8 h of incubation (Figure 4c and d). At the plasma cell stage, mostly mRNA for secreted μ chain is present. We determined the size of this mRNA to be 2.1 kb. In pre-B cells, an additional 2.35 kb mRNA encodes membrane μ chain and the

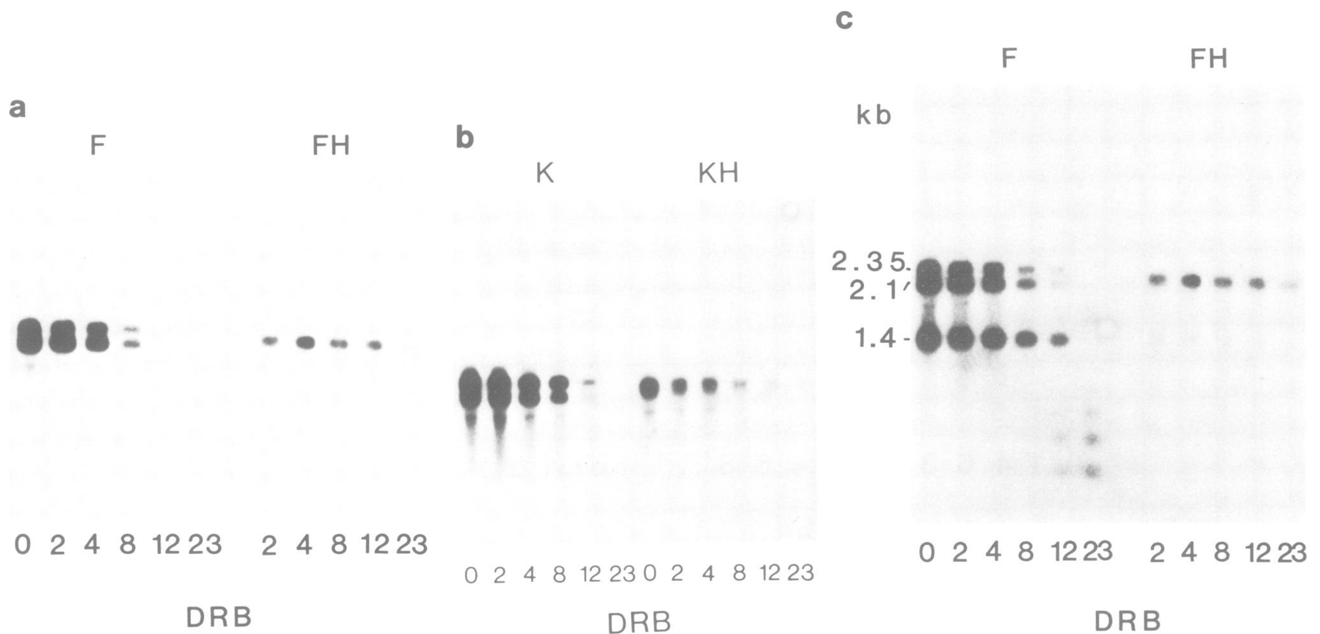

Fig. 5. Northern blot analysis of μ mRNA isolated from cells at various times after addition of DRB to the culture. Filters with bound mRNA were probed with nick-translated μ cDNA. (**a**) Pre-B cell line F and hybridoma FH; (**b**) pre-B cell line K and hybridoma KH; (**c**) blot shown in (a) rehybridized with a GAPDH probe. The numbers below the lanes indicate the number of hours after addition of DRB. 2.1 kb, mRNA for secreted μ chain; 2.35 kb, mRNA for membrane μ chain; 1.4 kb, mRNA for GAPDH. RNA standards used to determine the sizes of mRNA, given in kb: 28S from mouse lymphocytes, 5.0; 18S from mouse lymphocytes, 1.84; 26S from yeast, 3.39; 18S from yeast, 1.8; 23S from *Escherichia coli*, 2.9; 16S from *E.coli*,1.54; 7.5 kb RNA, 7.5; RNA ladder, 9.5, 7.5, 4.4, 2.5, 1.4, 0.3. The 1.4 kb band of the FH line is quite weak, because only one tenth of the mRNA, compared to F, was loaded. Longer exposure showed no degradation up to 12 h.

decay rate is the same for both species of mRNA.

Because actinomycin C intercalates into double-stranded nucleic acids, it might also stabilize mRNA, which has a secondary structure, including stretches of double-stranded RNA. If so, this cannot explain the difference in stability of the same mRNA in different cells. When we rehybridized a Northern blot with a histone 2b DNA probe, there was no mRNA left in the FH line after 1 h of incubation with actinomycin C (Figure 4e). Furthermore, Piechaczyk *et al.* (1985) and Dani *et al.* (1984) have reported that the half-life of c-*myc* mRNA was about the same whether determined with actinomycin or by following uridine incorporation rate until equilibrium. Thus, certainly not all species of mRNA, if any, are stabilized by this drug.

As an alternative to actinomycin C, we used 5,6-dichloro-1-β-D-ribofuranosyl benzimidiazole (DRB), a specific inhibitor of RNA polymerase II, which synthesizes mRNA (Zandomeni *et al.*, 1983). DRB affects initiation but not elongation or termination (Zandomeni *et al.*, 1983), enters cells rapidly and inhibits nuclear RNA synthesis in less than 15 min. Although synthesis of very short nuclear RNA molecules continues, DRB blocks mRNA labeling in the cytoplasm by more than 99% (Tamm *et al.*, 1984). Furthermore, few secondary effects of DRB have been reported (Tamm *et al.*, 1984). Khalili and Weinmann (1984) found the same half-life of actin mRNA in HeLa cells by using DRB in pulse-chase experiments with radioactively labeled precursors. Thus, for measurements of mRNA turnovers, DRB is an appropriate inhibitor of RNA synthesis. Like actinomycin C, DRB inhibited [^{3}H]uridine incorporation into RNA in lines F and FH 1 h after addition (not shown).

Even though DRB has a mechanism of action different from that of actinomycin C, mRNA stability was about the same in the presence of either drug (Figure 5a). The cells of the more than 30 clones we tested are viable 7 h after actinomycin, and they start to degenerate after 8 h. However, in the presence of DRB cells are viable for at least 12 h. Thus, we followed the μ mRNA decay up to 23 h. In clone FH, only at 23 h did we see a clear decay in the amount of μ mRNA (Figure 5a), while the decay of μ mRNA in clone F was much faster. For unknown reasons, the difference between K and KH was not as great with DRB (Figure 5b) as it was with actinomycin C.

It might be argued that the apparent long lifetime of μ mRNA in plasma cells is due to replenishment from the nuclear RNA pool by splicing and transport. This argument does not hold up to quantitative scrutiny, however, because the nuclear RNA pool in plasma cells is at least two orders of magnitude smaller than the pool of mRNA in the cytoplasm (Schibler *et al.*, 1978).

In conclusion, we have shown that the variation in the steady state level of μ mRNA in B cells of various differentiation stages cannot be attributed to differences in transcription rates, but to differences in μ mRNA stability. This difference in stability may not be peculiar to immunoglobulin mRNA, as it seems to apply also to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Over 4 h there is no detectable decay of mRNA encoding GAPDH in either pre-B or plasma cell lines (Piechaczyk *et al.*, 1985). However, a large part of GAPDH mRNA was degraded after 12 h in the pre-B line F, but not in line FH (Figure 5c). We also noted specific degradation products. It seems that an RNase with specificity for sequences of GAPDH mRNA is active, while the resulting mRNA fragments are quite stable. Such a phenomenon has also been reported in bacteria (Ö.Melefors and A.von Gabain, in press).

What could lead to the difference in mRNA stability in the various differentiation stages? Plasma cells have many

more ribosomes than pre-B cells, and efficient translation may protect mRNA in plasma cells from being degraded. Indeed, μ mRNA that cannot be fully translated may be quickly degraded (Baumann *et al.*, 1985).

Materials and methods

Cell culture and cell fusion

All cell lines were grown in RPMI 1640 supplemented with 10% fetal calf serum, 4 mM L-glutamine, 1000 U/ml of a penicillin–streptomycin solution, 0.05 mM β-mercaptoethanol and 1 mM sodium pyruvate in the presence of 5% CO_2 at 37°C.

Fusion of K cells to Ag8.653 was performed as described by Burrows *et al.* (1981). Because line K is not sensitive to hypoxanthine–aminopterin–thymidine (HAT) medium, a modification of the method of Wright and Hayflick (1975) was used to select against K cells that had not fused with the myeloma.

For Northern blot experiments, cells were grown at a density of 5 × 10^5 cells/ml. After counting, the cells were kept for 1 h at 37°C before the addition of DRB (Calbiochem), final concentration 0.1 mM from a stock solution of 100 mM in dimethyl sulfoxide, or actinomycin C1 (Boehringer Mannheim), final concentration 5 μg/ml from a stock solution of 10 mg/ml in dimethyl sulfoxide. At various times, 10^7 cells were removed, centrifuged, quick-frozen in liquid N_2 and stored at −70°C until RNA was isolated.

To measure the incorporation of uridine into RNA, 5 × 10^5 cells/ml were labeled with 50 μCi [5,6-^{3}H]uridine (Amersham, 50 Ci/mmol). For experiments in the presence of the drug, 5 μg/ml actinomycin C was added 75 min after the addition of [^{3}H]uridine. Triplicate samples (1 ml) were taken at indicated times and chilled on ice, and ^{3}H-labeled RNA was precipitated on glass fiber filters and counted.

DNA probes for nuclear run-on transcription assays

Double-stranded probes and plasmids were isolated by standard procedures, as follows.

μ cDNA probe: isolated *Pst*I fragment of pMKμ-1 made by M.Knapp. It covers the first three exons of Cμ (Cμ1, Cμ2, Cμ3) and 10 bp of the fourth exon (Cμ4).

Cμ34 probe: The plasmid pGGMμ3 is the plasmid pBR322 containing the genomic *Hin*dIII insert covering the Cμ3 and Cμ4 exons.

GAPDH probe: A 1.25 kb *Pst*I–*Xba*I fragment was isolated from the plasmid pRGAPDH-13 containing a cDNA insert (Fort *et al.*, 1985). The plasmid was a gift of K.Marcu.

Histone 2b probe: the H2b probe (Grandy *et al.*, 1982) was purchased from Oncor, Inc. (Gaithersburg, MD).

M13μ34 and M13μ43 probes: Single-stranded probes were constructed by cloning the fragment into M13mp19 (Messing, 1983). A 1.2 kb *Hin*dIII fragment of the plasmid pGGMμ3 was cloned into the *Hin*dIII site of M13 mp19 in both orientations. The diagnostic digests were done by simultaneous digestion with the enzymes *Bst*EII and *Bgl*II.

Single-stranded M13 DNA probes were isolated by standard procedures described in the sequencing manual of Bethesda Research Laboratory (1984). For binding of DNA probes to nitrocellulose (BA85, Schleicher and Schuell), 5 μg of *Bam*HI-digested pGGMμ3, or 0.5 μg of isolated double-stranded probes (both probes are designated Cμ34), and 0.5 μg of M13 single-stranded probes (designated 43 and 34) were dissolved in 100 μl TE/0.3 N NaOH, heated for 45 min at 60°C, cooled to room temperature, mixed with 100 μl 2 N NH_4 acetate (NH_4Ac), pH 7.0 and immediately dot-blotted onto nitrocellulose (prewetted in 1 N NH_4Ac) with a dot blot apparatus (Schleicher and Schuell). Wells were washed with 200 μl 1 N NH_4Ac; filters were dried under a heat lamp for 20 min and baked for 3 h at 80°C in a vacuum oven.

RNA analysis by Northern blotting and cytoplasmic dot hybridization of μ mRNA

Cytoplasmic RNA was isolated by a modification of Favaloro *et al.* (1980). Briefly, 10^7 cells were lysed in 0.5% NP40. The nuclei were removed by centrifugation in an Eppendorf cup at 12 000 *g* for 3 min at 5°C. The supernatant was digested with proteinase K (100 μg/ml) in the presence of 1% SDS, extracted five times with phenol/chloroform (1:1) and once with chloroform, and precipitated with ethyl alcohol. The precipitate was dissolved in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA). The concentration of RNA was determined by measuring the absorbance at 260 nm.

The same amount of RNA was applied to each lane of a 1.0% agarose gel containing 2.2 M formamide and run overnight using a constant current of 10–15 mA.

For cytoplasmic dot hybridization, RNA was spotted to nitrocellulose as described (Wabl and Burrows, 1984).

Hybridization and rehybridization were performed as described (Meinkoth and Wahl, 1984). Filters were processed with an Omniblot system (ABN) according to the manufacturer's instructions. Filters were washed consecutively with 500 ml of 2 × SSC (1 × SSC = 0.15 M NaCl/0.0015 M sodium citrate, pH 7.0),0.1% SDS at 48°C and with 1000 ml of 0.1 × SSC, 0.1% SDS at 63°C.

TCA precipitation of labeled RNA

Cells or nuclei were suspended in 100 μl of 40 mM EDTA, 0.2% SDS and precipitated with 5 ml of ice-cold 10% trichloroacetic acid (TCA), 10% saturated sodium pyrophosphate for 10 min. Precipitates were collected on glass fiber filters. The filters were washed twice with 5 ml of 10% TCA, once with 95% ethyl alcohol, dried and counted in a liquid scintillation mix (Ecolite) in a β scintillation counter.

In vitro transcription in isolated nuclei (nuclear run-on)

Nuclei from 10^8 cells were isolated as described by Marzluff and Huang (1984) with the following modifications. Cells were lysed in 0.08% Triton X-100 by 15 strokes in a Dounce homogenizer (Pestle B). Nuclei were pelleted through 2 M sucrose in an SW28 rotor (Beckman) at 20 000 r.p.m. for 1 h at 3°C. The nuclei were resuspended in nuclei freezing buffer (25% glycerol, 5 mM magnesium acetate, 0.1 mM EDTA, 50 mM Tris–HCl, pH 8.0) at a concentration of 2 × 10^8 nuclei/ml. The nuclei were quick-frozen in liquid N_2 and stored at −70°C.

The nuclear run-on reactions were performed with slight modifications as described (Linial *et al.*, 1985). In the transcription assays, 100 μl nuclei (2 × 10^7), 30 μl of 5 × run-on buffer [25 mM Tris–HCl, pH 8.0, 12.5 mM magnesium acetate, 750 mM KCl, 1 mM triphosphates of A, G and C, and 1 mM S-adenosyl-methionine (Chen-Kiang *et al.*, 1982)]), 15 μl [α-^{32}P]UTP (Amersham, 800 Ci/mmol, 40 mCi/ml) and 5 μl H_2O were incubated at 26°C for 10–12 min. Samples were then chilled on ice, 190 μl of DNase-mix (1 × run-on buffer, 100 μg tRNA/ml, 1 mM $CaCl_2$, 200 U DNase I/ml from Promega) was added, and the suspension was incubated for 5 min at 30°C. Nuclei were lysed by addition of 80 μl proteinase K mix (200 μg proteinase K/ml, 5% SDS, 50 mM Tris–HCl, pH 8.0, 50 mM EDTA) and digested for 1–2 h at 42°C. The mixture was extracted 2 × with phenol/chloroform (1:1) and 1 × with chloroform and precipitated with 2.5 volumes of ethyl alcohol for 1 h at −70°C. The precipitate was centrifuged for 30 min in a microcentrifuge, and the pellet was dissolved in 100 μl TE buffer and centrifuged through a prepared G50 column (Boehringer Mannheim). The eluate was digested again with 30 U of DNase I for 5 min at 30°C in 10 mM $MgCl_2$, 1 mM $CaCl_2$ and with proteinase K (100 μg/ml) in 0.1% SDS, 2 × extracted with phenol/chloroform (1:1) and 2 × with chloroform. RNA was precipitated with 1/2 volume 7.5 M NH_4Ac and 2.5 volumes of ethyl alcohol overnight at −20°C. The precipitate was centrifuged for 30 min at 5°C, the pellet dissolved in TE. The solution was made 0.1 N in NaOH and after 10 min on ice, Hepes was added to a concentration of 0.24 M. Two-and-one-half volumes of ethyl alcohol were then added and the solution was held overnight at −20°C. After centrifugation, the pellet was dissolved in 100 μl TE and 1 μl was TCA precipitated. The yield at this stage was 1–2 × 10^7 c.p.m./10^7 nuclei. In some experiments Sarkosyl (*N*-lauroyl sarcosine sodium salt; Sigma) was added.

Filters with the respective probes were prehybridized in 1 ml 50% formamide, 5 × SSC, 50 mM sodium phosphate, pH 7.0, 0.1% SDS, 1 mM EDTA, 100 μg/ml polyA and 250 μg/ml denatured salmon sperm DNA in polypropylene tubes overnight at 45°C under shaking. Hybridization was done for 4–5 days under the same conditions except that 20 mM sodium phosphate, pH 7.0, and 100 μg salmon sperm DNA was added. The input of ^{32}P-labeled RNA ranged between 1–8 × 10^6 c.p.m./ml. The hybridization mix was overlaid with 200 μl light paraffin oil to prevent evaporation. After hybridization, the filters were washed twice in 2 × SSC/0.1% SDS at 48°C for 30 min and three times in 0.1 × SSC, 0.1% SDS for 20 min, each at 65°C. The filters were autoradiographed for 1–3 days at −70°C.

Acknowledgements

We thank Alexander von Gabain and Charles Steinberg for discussions, Rudolf Grosschedl for help with the manuscript, Mary McKenney for editing it and Michelle Bloom for typing it. This work was supported by the Office

of Health and Environmental Research, US Department of Energy, contract no. DE-AC03-76-SF01012, and by the National Institutes of Health grant 1 R01 GM37699-01A1. It is taken from a thesis (in German) submitted by Hans-Martin Jäck to the University of Tübingen in partial fulfillment of the requirements for a Ph.D. degree.

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Received on December 10, 1987; revised on February 1, 1988