Changes in non-histone nuclear proteins during postnatal myocardial development

George JACKOWSKI and Choong-Chin LIEW

Department of Clinical Biochemistry and Medicine, Banting Institute, University of Toronto, Toronto, Ontario
M5G 1L5, Canada

(Received 16 August 1982/Accepted 2 September 1982)

Myocardial-cell nuclei isolated from ventricles of rats between 4 days and 15 months of age showed a progressive loss of DNA-synthetic activity *in vitro*. Correlated with this loss is the differential appearance of three major groups of non-histone nuclear proteins. Group a, with pI 5.7–6.5 and M_r 67 000, appeared in detectable amounts in rats more than 10 days of age, whereas group b, consisting of proteins with pI 6.8–7.2 and M_r 45 000, appeared in rats older than 33 days. The third group (group c), with pI 6.5–8.5 and M_r 32 000–42 000, was present throughout postnatal development, but the amount of these proteins appeared to be greater in the 10-day-old rat than in the 15-month-old rat. All groups of these specific proteins were localized within the nucleus and did not reflect cytoplasmic constituents. Furthermore, the amino acid compositions of these polypeptides were also analysed and compared with those of actin and tubulin.

Myocardial-cell DNA synthesis and proliferation in the rat heart declines rapidly, and by the third week of postnatal development essentially ceases (Claycomb, 1973, 1975, 1976, 1978; also reviewed by Dow *et al.*, 1981). As the heart grows in size, fully differentiated myocardial cells lose the ability to replicate their own DNA, undergo mitosis, and proliferate, whereas the non-myocardial cells, mainly consisting of interstitial and endothelial cells, retain their capacity to proliferate under the appropriate stimulus. To date, little is known about the mechanisms regulating the repression of myocardial DNA synthesis and cellular proliferation; however, there is evidence that genetic expression in eukaryotic cells may be regulated by specific DNA-associated proteins (Allfrey *et al.*, 1971; Stein *et al.*, 1974; MacGillivray & Rickwood, 1974; MacGillivray *et al.*, 1978; Wang *et al.*, 1976; Bekhor, 1978). In the last few years we have been involved in identifying and characterizing DNA-associated proteins in various tissues by two-dimensional polyacrylamide-gel electrophoresis (Suria & Liew, 1974a; Liew & Chan, 1976; Chan & Liew, 1977, 1979; Jackowski *et al.*, 1976; Liew, 1979). We have also employed this technique to identify myocardial nuclear protein changes associated with genetically determined cardiomyopathy in the Syrian hamster and in the human (Liew & Sole, 1978a,b; Liew

et al., 1980). By using this technique, we proceeded to investigate age-related changes in specific myocardial nuclear proteins during postnatal development.

Experimental procedure

All chemicals used in the present study were of reagent grade. Experiments were carried out on rats (Wistar strain) aged 4 days to 15 months.

Isolation of myocardial cells and nuclei

Myocardial cells were isolated from rat hearts by a modification of the method of Glick *et al.* (1974). The intact myocardial cells isolated were washed repeatedly with phosphate buffer before the isolation of myocardial nuclei. The method used to release the myocardial nuclei from the cells was described previously (Jackowski & Liew, 1980a,b).

Isolation of ventricular nuclei

Rats were killed by decapitation. All subsequent operations were carried out at 4°C. Hearts were removed and rinsed in ice-cold buffer (MA buffer), consisting of 0.25 M-sucrose/10 mM-Tris/HCl (pH 7.4)/3 mM-MgCl₂/0.1 mM-PMSF. After the aorta and atria were removed, the ventricular nuclei were isolated by buoyant-density centrifugation (Jackowski & Liew, 1980b). This procedure allows for the isolation of an enriched homogeneous

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; DNAase, deoxyribonuclease; hnRNP, heterogeneous ribonucleoprotein.

population of biologically active myocardial nuclei (Liew *et al.*, 1982).

Extraction of nuclear proteins

Total nuclear proteins were extracted from rat nuclei at different ages. The nuclei were washed twice with 0.14 M-NaCl/0.2 mM-PMSF, then suspended in 0.1 M-Tris/HCl (pH 8.4)/0.1 mM-EDTA/0.14 M-2-mercaptoethanol (TEM buffer) and extracted with TEM buffer/phenol (1:1, v/v) (Shelton & Allfrey, 1970; Teng *et al.*, 1971). We have used several extraction procedures to extract nuclear proteins such as urea/guanidinium chloride, SDS, TEM/phenol (Suria & Liew, 1974a,b; LeStourgeon & Beyer, 1977; Liew, 1979) and found them to extract essentially the same class of nuclear proteins. We found the TEM buffer/phenol extraction method (see review by Liew, 1979) to be the method of choice. If nuclei were extracted with TEM buffer/phenol without prior removal of histones (by not extracting nuclei with 0.25 M-HCl), the recovery of total nuclear proteins was greater than 85%. This is comparable with the method of LeStourgeon & Beyer (1977), who used SDS/phenol.

Isolation and fractionation of cardiac sarcoplasmic-reticulum proteins

Cardiac sarcoplasmic-reticulum vesicles were prepared from adult rat heart (50-day-old) ventricles by the procedure of Hariyaga & Schwartz (1969). Sarcoplasmic-reticulum proteins were solubilized in TEM buffer/phenol (Suria & Liew, 1974b).

Polyacrylamide-gel electrophoresis

The non-histone nuclear proteins were extracted with TEM buffer/phenol after the initial removal of the acid-soluble proteins (mainly histones) with 0.25 M-HCl. These nuclear proteins were dialysed in urea buffer containing 0.02 M-glycine to prevent possible carbamylation during dialysis (MacGillivray & Rickwood, 1974). The nuclear proteins were then fractionated according to their isoelectric point in the first dimension and subsequently resolved according to their M_r in the second dimension by SDS/polyacrylamide-slab-gel electrophoresis by the method used previously (Jackowski & Liew, 1980b).

Determinations of protein, DNA, M_r and pH gradients of the gel

DNA was determined by the method of Burton (1956). The protein sample was precipitated by 10% (w/v) trichloroacetic acid and the determination was carried out by the method of Lowry *et al.* (1951). Relative molecular masses (M_r) of nuclear proteins were estimated by using standard protein markers: human carbonic anhydrase (M_r 29 000), ovalbumin (45 000), bovine serum albumin (67 000) and phosphorylase α (91 000). The pH gradient within the gel

was determined by slicing the gel into 1.5 mm slices, incubating the individual slices for 2 h in 1 ml of degassed 10 mM-KCl and then measuring the pH.

Amino acid analysis

Total non-histone nuclear proteins were fractionated by two-dimensional polyacrylamide-gel electrophoresis. After the two-dimensional electrophoresis the individual proteins were revealed by placing the gel into 10 vol. of 4 M-sodium acetate for 30 min at room temperature. Spots corresponding to specific non-histone proteins [group a (pI 5.7–6.5, M_r 67 000), group b (pI 6.8–7.2, M_r 45 000), group c_1 (pI 6.5–8.5, M_r 37 000), c_2 (pI 6.5–8.5, M_r 34 000), actin (pI 5.2, M_r 45 000)] were excised and collected from 14 replicate slab gels (each gel containing approx. 500 μ g of total non-histone proteins). The specific non-histone proteins were recovered from the gel slices by electrophoretic elution into dialysis bags (Stephens, 1975). Upon completion, the electrophoretically eluted samples were dialysed against cold water and freeze-dried in preparation for amino acid analysis. A Beckman model 120C amino acid analyser was used to analyse the proteins after their hydrolysis in 6 M-HCl at 110°C for 24 h.

DNA synthesis in vitro by isolated nuclei

Non-Triton-treated ventricular cardiac nuclei were used for DNA synthesis *in vitro*. The assay measured the incorporation of [3 H]dTMP by ventricular nuclei into acid-insoluble DNAase-sensitive products. The reaction mixture, in a total volume of 0.5 ml, containing 100 mM-Tris/HCl, pH 7.5, 0.1 mM-dATP, 0.1 mM-dGTP, 0.1 mM-dCTP, 238 pmol of [3 H]dTTP, 6 mM-MgCl₂, 4 mM-ATP and 3×10^6 nuclei (Claycomb, 1977). The nuclei in this mixture were incubated at 37°C for periods up to 40 min after a 5 min preincubation period. At given times 50 μ l of this mixture was spotted on Whatman filter paper (32 ET) and precipitated using 10% (w/v) trichloroacetic acid/2% (w/v) sodium pyrophosphate at 4°C. The filter paper was washed in the same trichloroacetic acid/sodium pyrophosphate solution for 30 min, followed by two 15 min washes in 95% (v/v) ethanol, a final rinse in diethyl ether, dried at 100°C and counted for radioactivity in a toluene/Omniflor scintillation cocktail. The results were corrected for zero-time radioactivity. A 50 μ l sample of this DNA assay mixture was obtained to determine the exact number of nuclei per sample.

Results

Two-dimensional polyacrylamide-gel electrophoresis of non-histone nuclear proteins

Recovery of ventricular nuclei from all ages of rats is presented in Table 1. Younger rats had a higher recovery of nuclei than older rats; however,

Table 1. *Recovery of ventricular cardiac nuclear DNA from the homogenate*

Details of the isolation procedure are described in the Experimental procedure section.

Age of rat	DNA ($\mu\text{g/g}$ of ventricle)		Recovery (%)
	Homogenate	Nuclei	
10 days	5900	1686	28.6
20 days	3424	887	25.9
33 days	1529	412	26.9
50 days	1180	267	22.6
15 months	939	186	19.8

the yield of non-histone nuclear proteins by the method of TEM buffer/phenol extraction was similar in both the young and older rat heart. Fig. 1 shows typical time courses of DNA synthesis by ventricular nuclei from 4-day-, 10-day-, 30-day- and 90-day-old rats. The rate and extent of DNA synthesis *in vitro* decreases progressively during postnatal development.

The age-related changes in cardiac nucleoproteins after postnatal development were resolved by two-dimensional polyacrylamide-gel electrophoresis. Fig. 2 compares the electrophoretic patterns derived from 10-day-old (1), 20-day-old (2), 33-day-old (3) and 15-month-old rats (4). Visual inspection indicates that three main groups of ventricular non-histone nuclear proteins appear differentially with postnatal development. Group a, consisting of proteins with isoelectric point (pI) 5.7–6.5 and M_r 67000, appears in detectable amounts in neonatal animals more than 10 days of age, whereas group b, consisting of proteins with pI 6.8–7.2 and M_r 45000, appears in neonatal animals older than 33 days.

In both groups of proteins (a and b), their respective maximum amounts are reached by 33 days and thereafter remain fairly constant with age. In group c, the major proteins were found to have pI values between 6.5 and 8.5 with M_r from 32000 to 42000. These proteins are present throughout postnatal development; however, their amounts appear to be greater in the 10-day-old neonate than in the 15-month-old rat. The differences observed for each age of rat were reproducible and the patterns shown in Fig. 2 are representative of several experiments.

Localization and analysis of the specific non-histone nuclear proteins

In order to investigate whether these three groups of nuclear proteins (a, b and c), which appear differentially with postnatal development, are truly specific to the myocardial cell, we decided to isolate

Fig. 1. $[^3\text{H}]\text{dTMP}$ incorporation into DNA of isolated ventricular cardiac nuclei prepared from rats of various ages

DNA synthesis was examined in isolated non-Triton X-100 treated ventricular cardiac nuclei obtained from 4-day-old, 10-day-old, 33-day-old and 90-day-old rats. Nuclei (3×10^6) were incubated with $[^3\text{H}]\text{dTMP}$. Aliquots were removed at the indicated times and acid-precipitable radioactivity was determined.

pure myocardial cells and their nuclei using enzyme digestion. Nuclear proteins extracted from purified myocardial nuclei fractionated on the two-dimensional gel system are shown in Fig. 3. All three groups (a, b and c) of nuclear proteins which appear differentially with postnatal development are present.

The nuclear proteins (groups a, b and c) that appear differentially with postnatal development may be nuclear in origin or may possibly reflect contaminating sarcoplasmic-reticulum proteins, which have been shown to increase with postnatal development. In order to resolve their origin, the sarcoplasmic-reticulum proteins from 50-day-old-rat ventricles were fractionated by two-dimensional polyacrylamide-gel electrophoresis (Fig. 4). The

Fig. 2. *Two-dimensional polyacrylamide-gel electrophoresis*

Nuclear proteins ($400\mu\text{g}$) obtained from ventricular tissue of 10-day-old (a), 20-day-old (b), 33-day-old (c) and 15-month-old (d) rats were analysed by two-dimensional polyacrylamide-gel electrophoresis. Arrowed areas indicate regions of particular interest referred to in the text.

Fig. 3. *Two-dimensional polyacrylamide-gel electrophoresis of myocardial cell nuclear proteins*

Nuclear proteins ($250\mu\text{g}$) from purified myocardial cell nuclei isolated by enzyme digestion were analysed by two-dimensional polyacrylamide-gel electrophoresis.

Fig. 4. *Two-dimensional polyacrylamide-gel electrophoresis of sarcoplasmic-reticulum proteins*

Sarcoplasmic-reticulum proteins ($400\mu\text{g}$) obtained from adult ventricles were analysed by gel electrophoresis. Arrowed areas indicate regions of particular interest referred to in the text.

electrophoretic patterns show a heterogeneous group of sarcoplasmic-reticulum proteins. The specific nuclear proteins of group a (pI 5.7–6.5, M_r 67 000) and group c (pI 6.5–8.5, M_r 32 000–42 000) could not be detected; however, a specific group of sarcoplasmic proteins which have the same pI and M_r as nuclear proteins belonging to group b (pI 6.5–7.2, M_r 45 000) were present (Fig. 4). Since other specific sarcoplasmic-reticulum proteins (arrowed pI 6.4–7.8, M_r 38 000; pI 4.8, M_r 50 000) found in larger amounts in cardiac sarcoplasmic reticulum are not found in the nucleus (Fig. 2), the occurrence of this group of nuclear proteins is not likely the result of sarcoplasmic-reticulum contamination.

Furthermore, in order to investigate whether these groups of nuclear proteins (a, b and c) were contractile proteins, ventricular homogenates from 10-day-old neonates and 50-day-old rats were also analysed by two-dimensional polyacrylamide-gel electrophoresis. As shown in Fig. 5, the electrophoretic patterns of whole heart tissue homogenate (consisting mainly of cytoplasmic proteins) of the 10-day-old neonate and the 50-day-old rat were similar, except for certain quantitative differences. Non-histone nuclear proteins belonging to group a (pI 5.7–6.5, M_r 67 000) and group c (pI 6.5–8.5, M_r 32 000–42 000) are specifically located in the nucleus and could barely be detected in either the tissue

Fig. 5. Two-dimensional polyacrylamide-gel electrophoresis of cytoplasmic proteins

Cytoplasmic proteins (400 μ g) obtained from ventricular homogenate as described in the Experimental procedure section were analysed by two-dimensional polyacrylamide-gel electrophoresis. (a) 10-day-old rats; (b) 50-day-old rats.

Fig. 6. Two-dimensional polyacrylamide-gel electrophoresis of nuclear proteins

(a) represents nuclear proteins (250 μ g) obtained from purified nuclei isolated from a mixture of 15-day-old neonatal animal crude nuclei mixed with adult sarcoplasmic-reticulum fractions as described in the text. (b) represents nuclear proteins obtained from adult ventricular nuclei.

Table 2. *Amino acid compositions of specific nuclear non-histone proteins recovered*
No corrections were made for hydrolytic losses.

Amino acid	pI ... M_r ...	Composition (mol/100 mol)				
		Actin	Group a	Group b	Group c ₁	Group c ₂
		5.2 45 000	5.7–6.5 67 000	6.8–7.2 45 000	6.5–8.5 37 000	6.5–8.5 34 000
Aspartic acid (A)		8.1	9.4	11.8	7.0	8.9
Threonine		5.2	2.8	2.5	5.0	3.1
Serine		12.0	4.2	3.2	15.4	5.6
Glutamic acid (A)		15.5	18.7	17.7	12.0	16.5
Proline		0.0	2.7	3.1	0.8	2.4
Glycine		23.5	22.7	22.4	26.3	24.4
Alanine		8.2	8.5	4.6	10.9	9.6
Half-cystine		0.7	0.0	0.7	1.3	0.0
Valine		3.8	3.9	5.3	4.0	4.6
Methionine		0.0	0.0	0.0	0.0	1.0
Isoleucine		4.3	3.0	4.5	2.5	2.6
Leucine		5.6	7.0	7.8	3.8	4.3
Tyrosine		0.8	3.2	2.0	1.4	2.7
Phenylalanine		1.3	2.6	1.6	0.9	2.9
Histidine (B)		2.1	1.8	2.6	2.7	2.4
Lysine (B)		6.4	6.4	5.8	4.7	6.4
Arginine (B)		2.5	3.2	4.1	1.8	2.5
Acidic (A)/basic (B)		2.1	2.5	2.4	2.1	2.2

homogenate of the 10-day-old neonate or the 50-day-old rat. Group-b nuclear proteins (pI 6.8–7.2, M_r 45 000), however, are found in the whole heart tissue homogenate in the 50-day-old rat and much less in the 10-day-old neonate, indicating that these proteins may be cytoplasmic proteins or are released into the cytoplasm during homogenization.

Finally, in order to elucidate whether proteins belonging to group b represent a specific group of cytoplasmic proteins associated with the nucleus, the 8000 g supernatant (containing essentially microsomes) from adult (70-day) tissue homogenate was mixed and homogenized with crude nuclei from 15-day-old neonates. Nuclei from this mixture were subsequently isolated and purified. As shown in Fig. 6, nuclear proteins isolated from these nuclei did not reveal any specific contaminant of the sarcoplasmic-reticulum protein (pI 6.8–7.2, M_r 45 000). Consequently, proteins of group b (arrowed, pI 6.8–7.2, M_r 45 000) seem to be localized in two specific compartments (nucleus and sarcoplasmic reticulum).

Amino acid analysis (Table 2) indicated that the acidic-to-basic ratio in groups a, b and c is greater than 2.0. Given that these proteins (a, b and c) appear to be focused in the neutral-to-basic region of the isoelectric-focusing gel, it seems likely that a large proportion of acidic residues could be present as asparagine and glutamine, since during acid hydrolysis all the asparagine and glutamine would be

converted into aspartic acid and glutamic acid. The high levels of glycine seen in all the proteins analysed may reflect a trace amount of glycine from the running buffer left behind even after exhaustive dialysis against water. All three groups of non-histone proteins had distinctly different amino acid compositions when compared with those of actin or tubulin. Group-c nuclear protein has a similar amino acid composition to that of protein associated with heterogeneous ribonucleoprotein particles. Groups a and b proteins are shown to be specifically associated with postnatal heart development.

Discussion

One of the most remarkable phenomena in the developing myocardium is that shortly after birth the myocardial cells cease to divide, thus ending proliferative growth. It has been demonstrated by Claycomb (1973, 1975, 1977) that there is a close temporal correlation between the age-dependent decline of mitosis and decreased activities of the enzymes associated with DNA synthesis.

In the present study, temporally correlated with progressive loss of DNA-synthetic activity *in vitro* is the differential appearance of specific nuclear proteins. We have been able to identify three major groups of myocardial non-histone nuclear proteins which appear differentially with postnatal development. Group a (pI 5.7–6.5, M_r 67 000) and group b (pI 6.8–7.2, M_r 45 000) were found to increase

differentially with postnatal development. Group a is more predominant in muscle-cell nuclei than in nuclei isolated from brain, kidney, or liver (Liew, 1979; Fraser *et al.*, 1979), whereas group b appears to be tissue-specific for the heart muscle nuclei and is not detectable in the brain, kidney, or liver (Liew, 1979). Group c (pI 6.5–8.5, M_r 32 000–42 000) are found in all cell types studied (Liew, 1979) and are associated with a group of nuclear proteins involved in the transport and processing of nuclear mRNA (hnRNP) (Georgiev, 1971; Karn *et al.*, 1977; Suria & Liew, 1979). It appears that this fraction of proteins (group c) are greater in 10-day-old rats than in 15-month-old rats and remains fairly constant within these two ages. This is in agreement with what is known of the development of the myocardium: before birth and during the first few weeks after birth, the myocardial cells are actively involved in RNA and protein synthesis (Zak, 1975).

The recovery of isolated nuclei is much less in the adult rat heart as a result of the significant growth of myofibrils, which increase the structural tenacity of the myocardial cells. However, once the nuclei were isolated, the recovery of non-histone nuclear proteins was similar in both young and adult rat hearts. It is unlikely that our observations on the qualitative and quantitative changes in non-histone nuclear proteins during postnatal cardiac development were the result of an artefact due to traces of cytoplasmic proteins contaminating our nuclear preparation. We have ruled out this possibility by cross-examination of the cellular components between the young and adult rat hearts (Figs. 4–6). It is also believed that some non-histone nuclear proteins are not extractable in phenol buffer (Suria & Liew, 1974a). Thus variability in extractability could possibly give rise to some of the quantitative differences observed. However, if this is true, then variation in extractability cannot explain why the same proteins are preferentially solubilized in young or old ventricular nuclei while other proteins remain qualitatively and quantitatively similar. Furthermore, the amino acid compositions of the three polypeptides are distinctively different from that of actin or tubulin.

In conclusion, we have demonstrated in the present investigation that three major groups of myocardial nuclear non-histone proteins appear differentially with postnatal development. The mechanism(s) involved in the interaction of these nuclear proteins and DNA synthesis have yet to be studied.

The generous support from the Medical Research Council (Canada), Ontario Heart Foundation of Canada and U.S. Public Health Service (grant no. HL 18224-01) is greatly acknowledged. G. J. is a Doctoral Fellow of the Ontario Heart Foundation.

References

- Allfrey, V. G. (1971) in *Histones and Nucleohistones* (Phillips, D. M. P., ed.), pp. 241–294, Plenum Press, New York
- Bekhor, I. (1978) in *The Cell Nucleus*, vol. 5 (Busch, H., ed.), pp. 137–166, Academic Press, New York
- Burton, K. (1956) *Biochem. J.* **62**, 315–323
- Chan, P. K. & Liew, C. C. (1977) *Can. J. Biochem.* **55**, 847–855
- Chan, P. K. & Liew, C. C. (1979) *Biochem. J.* **183**, 147–156
- Claycomb, W. C. (1973) *Biochem. Biophys. Res. Commun.* **54**, 715–720
- Claycomb, W. C. (1975) *J. Biol. Chem.* **250**, 3229–3235
- Claycomb, W. C. (1976) *J. Biol. Chem.* **251**, 6082–6089
- Claycomb, W. C. (1977) *Dev. Biol.* **61**, 245–251
- Claycomb, W. C. (1978) *Biochem. J.* **171**, 289–298
- Dow, J. W., Harding, N. G. L. & Powell, T. (1981) *Cardiovasc. Res.* **15**, 483–514
- Fraser, I. H., Gordon, A. S. & Liew, C. C. (1979) *J. Neurol. Sci.* **42**, 303–316
- Georgiev, G. P. (1971) *Adv. Cell. Biol.* **2**, 47–110
- Glick, M. R., Burns, A. H. & Reddy, W. J. (1974) *Anal. Biochem.* **61**, 32–42
- Hariyaga, S. & Schwartz, A. (1969) *Circ. Res.* **25**, 781–794
- Jackowski, G. & Liew, C. C. (1980a) *Anal. Biochem.* **102**, 321–325
- Jackowski, G. & Liew, C. C. (1980b) *Biochem. J.* **188**, 363–373
- Jackowski, G., Suria, D. & Liew, C. C. (1976) *Can. J. Biochem.* **54**, 9–14
- Karn, J., Vidali, G., Boffa, V. C. & Allfrey, V. G. (1977) *J. Biol. Chem.* **252**, 7307–7322
- LeStourgeon, W. M. & Beyer, A. L. (1977) *Methods Cell Biol.* **16**, 387–406
- Liew, C. C. (1979) *Recent Adv. Genet. Res.* **2**, 153–186
- Liew, C. C. & Chan, P. K. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 3458–3462
- Liew, C. C. & Sole, M. J. (1978a) *Circ. Res.* **42**, 628–636
- Liew, C. C. & Sole, M. J. (1978b) *Circ. Res.* **42**, 637–643
- Liew, C. C., Sole, M. J., Silver, M. D. & Wigle, E. D. (1980) *Circ. Res.* **46**, 513–519
- Liew, C. C., Jackowski, G., Ma, T. & Sole, M. J. (1982) *Am. J. Physiol.* in the press
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- MacGillivray, A. J. & Rickwood, D. (1974) *Eur. J. Biochem.* **41**, 181–190
- MacGillivray, A. J., Johnston, C., MacFarlane, R. & Rickwood, D. (1978) *Biochem. J.* **175**, 35–46
- Shelton, K. R. & Allfrey, V. G. (1970) *Nature (London)* **228**, 132–134
- Stein, G. S., Spelsberg, T. C. & Kleinsmith, L. J. (1974) *Science* **183**, 817–824
- Stephens, R. E. (1975) *Anal. Biochem.* **65**, 369–379
- Suria, D. & Liew, C. C. (1974a) *Can. J. Biochem.* **52**, 1143–1153
- Suria, D. & Liew, C. C. (1974b) *Biochem. J.* **137**, 355–362

- Suria, D. & Liew, C. C. (1979) *Can. J. Biochem.* **57**, 32–42
- Teng, C. S., Teng, C. T. & Allfrey, V. G. (1971) *J. Biol. Chem.* **246**, 3597–3609
- Wang, S., Chiu, J. F., Klyszejko, S., Fujitani, H., Hnilica, L. S. & Ansevin, A. T. (1976) *J. Biol. Chem.* **251**, 1471–1475
- Zak, R. (1975) *Circ. Res.* **35** (Suppl. 2), 17–26