RNA transcription in myocardial-cell nuclei during postnatal development

A study establishing an assay system for transcription *in vitro*

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A system for RNA transcription *in vitro* was established in order to determine the relative rate of RNA synthesis in neonatal and adult rat myocardial cells. This assay system optimizes the incorporation of [³H]UMP into RNA by using 3.5×10^7 myocardial-cell nuclei, and minimizes RNA degradation for at least 1 h in transcription *in vitro*, by the addition of human placental RNAase inhibitor. A 100 % increase in the incorporation of [³H]UMP into myocardial-cell RNA was found on addition of this inhibitor. Myocardial-cell nuclei derived from 5-, 10-, 15-, 20-, and > 100-day-old rat hearts indicated that there is a progressive decrease in RNA synthesis with age. A 3-fold increase in RNA synthesis in 5-day-old myocardial cell nuclei as compared with 20-day-old rat heart was found. RNA synthesis in the adult myocardial cell nuclei decreased more than 10-fold in comparison with the 5-day-old newborn. The incorporation of [³H]UMP into rat liver nuclear RNA was 3-fold greater than in the myocardial-cell nuclear RNA, even when compared with the highly active transcription of 12-day-old heart nuclei. In order to determine the relationship between total RNA synthesis and the extent of specific gene expression in myocardial-cell nuclei during development, two distinct cDNA probes were used for Northern-blot analysis. Our results indicate that myosin-heavy-chain gene expression is remarkably decreased with age, whereas the 'housekeeping' gene is continually expressed independently of age.

INTRODUCTION

Cardiac tissue is composed of two ontogenically distinct cell types which differ in their DNA-synthesis properties. The non-myocardial cells, which are composed mainly of fibroblasts and other cell types not myocardial in origin, maintain mitotic competency throughout life. In contrast, the myocardial cells, which contain the force-generating contractile components, lose mitotic competency approx. 21 days after postnatal development and, after this period, enlarge by the hypertrophy of pre-existing muscle cells (Alpert & Mulieri, 1986; Bugaisky & Zak, 1979; Claycomb, 1973, 1979). The myocardium therefore undergoes two distinct phases of developmental myogenesis: (i) an early initial phase in which the production of large muscle-specific proteins, such as myosin heavy chain, is required for the rapid hyperplastic growth of the myocardial cells (0–21 days), and (ii) a second, later, phase in which myocardial repair synthesis must be accommodated.

The processes of hyperplasia and hypertrophy in the developing myocardial cell are most likely explained by mechanisms regulating nucleic acid synthesis (DNA and RNA) at the level of the nucleus. Previous reports have indicated that *in vivo* and in the isolated perfused rat heart (two models which, by design, include the synthesis capacities of both myocardial and non-myocardial cells), there is a progressive decrease in RNA synthesis with age (Meerson *et al.*, 1978; McCully *et al.*, 1983; Liew *et al.*, 1983a). It has also been shown that DNA synthesis decreases in the newborn from a relatively high rate at 4 days of age to essentially maintenance values at 50 days (Claycomb, 1973, 1979; Jackowski & Liew, 1980).

Evidence suggests that the mechanisms governing the phenotypic and developmental expression of the myosin-heavy-chain genes are regulated primarily at the transcriptional level (Chizzonite & Zak, 1984; Flink *et al.*, 1979; Hoh *et al.*, 1978, 1979; Klotz *et al.*, 1981; Lompre *et al.*, 1979; Saidapet *et al.*, 1982; Sinha *et al.*, 1982; Umeda *et al.*, 1986). Therefore it would be of interest to determine the myocardial-cell nuclear control mechanisms which govern the developmental expression of proteins such as the myosin-heavy-chain gene during periods of mitotic activity (0–21 days of age) before the advent of external or intrinsic myocardial stress. In order to approach the elucidation of these mechanisms, we now describe a transcriptional system *in vitro* which has enabled us to determine the relative rate of transcriptional activity from the early newborn (5–20 days of age), when mitotic activity is relatively high, to adulthood (> 100 days), a period of mitotic quiescence. In addition, we have used a molecular approach in order to investigate the relative cardiac myosin-heavy-chain nuclear RNA content during the process of mitotic down-regulation in comparison with the nuclear RNA content of a non-specific general 'housekeeping' gene.

MATERIALS AND METHODS

Animals

Late-pregnant rats (Wistar strain) were obtained from Woodlyn Laboratories, Guelph, Ontario, Canada. All animals were fed with Purina Brand Laboratory Chow and water *ad libitum*. Newborn rats dated from the

morning of birth were removed from the mother just before experimental use.

Transcription *in vitro*

Myocardial nuclei were isolated by the non-enzymic procedure as described by Jackowski & Liew (1980), with the addition of 10 mM- β -mercaptoethanol to all solutions.

The concentration of myocardial nuclei used for transcription *in vitro* was determined by using a Coulter Counter (model ZF) with a 100 μ m orifice, aperture current of 0.5 mA, amplification 0.707 and threshold 14.

Transcription *in vitro* was performed essentially as described by Manley *et al.* (1980). Nuclei (3.5×10^7) were suspended in a 200 μ l reaction mixture containing 12 mM-Hepes/NaOH (pH 7.9), 7 mM-MgCl₂, 60 mM-KCl, 0.2 mM-EDTA, 1.3 mM-dithiothreitol, 10% (v/v) glycerol, 750 μ M each of ATP, CTP and GTP, 50 μ M-UTP, 7 μ M-[³H]UTP (21.7 Ci/mmol) and 1 unit of human placental RNAase inhibitor (Bolton Biologicals, St. Paul, MN, U.S.A.)/ μ l. The reaction mixture was incubated in an RNAase-free silanized glass culture tube (12 mm $\times$ 75 mm) at 26 °C for 1 h. Samples (10 μ l, 1.75×10^6 nuclei) were precipitated by ice-cold 10% (v/v) trichloroacetic acid and collected on to GFC filters (Millipore). The [³H]UMP incorporation into RNA was determined by liquid-scintillation counting. All results were corrected for zero-time points.

Isolation of myocardial nuclear RNA

Myocardial nuclear RNA was isolated by the hot-SDS/phenol method (Penman, 1966). RNA from myocardial nuclei was fractionated by electrophoresis on a 1%-agarose gel containing 3% (v/v) formaldehyde, 20 mM-Mops (pH 7.4) and 1 mM-EDTA (Lehrach *et al.*, 1977); 28 S and 18 S rRNA were used as standard markers. Fractionated RNA was detected by ethidium bromide staining, then transferred to nitrocellulose paper by the method described by Southern (1975).

Hybridization with myosin-heavy-chain and 'housekeeping' cDNA probes

Fractionated RNA was hybridized with pCMHC21, a cardiac myosin-heavy-chain gene cDNA probe which possesses homologous α and β coding sequences (Mahdavi *et al.*, 1982), and the cDNA probe pA50, a 'housekeeping' gene present in all tissues (Garfinkel *et al.*, 1982). (Both probes were kindly give by Dr. V. Mahdavi and Dr. B. Nadal-Ginard, Harvard Medical School, Boston, MA, U.S.A.). All cDNA probes were labelled by the random-primer-labelling method as described by Feinberg & Vogelstein (1984).

Quantitative analysis of 'Northern' hybridization

To determine the consistency of signal duplication, Northern blots were scanned with a BioMed (Fullerton, CA, U.S.A.) scanning laser densitometer. The integrals for each blot were calculated by the BioMed computer 1D analysis program.

RESULTS

Transcription *in vitro*

Freshly isolated myocardial-cell nuclei were used immediately for transcription *in vitro* at 26 °C for a duration of 1 h; however, transcription may be extended

Fig. 1. Effects of nuclear concentration on transcription *in vitro*

Nuclei (■, 3.5×10^7 ; ●, 7.0×10^7 ; ▲, 10.5×10^7) were incubated in parallel reaction mixtures (200 μ l) containing human placental RNAase inhibitor (1 unit/ μ l) and [³H]-UMP at 26 °C. Samples were removed after 60 min of incubation and the [³H]UMP incorporation, normalized to 1.75×10^6 nuclei, was measured by liquid-scintillation counting. All points shown represent the mean and variance of four to six experiments.

for at least 1 h more. To optimize RNA-transcription efficiency and reproducibility, various concentrations of nuclei preparations were assessed (3.5×10^7 – 10.5×10^7 nuclei/200 μ l of reaction mixture). For optimal [³H]UMP incorporation, 3.5×10^7 nuclei/200 μ l of mixture should be used; greater than 7.0×10^7 nuclei/200 μ l gave less than maximal incorporation into RNA (Fig. 1). Nuclear concentrations of less than 3.5×10^7 nuclei/200 μ l of reaction mixture were investigated previously and also found to give less than maximal [³H]UMP incorporation into RNA (Liew & McCully, 1986).

Myocardial and liver nuclear transcription

In order to provide a comparison for nuclear transcriptional capacity, we have compared the rates of [³H]UMP incorporation in myocardial-cell nuclei and liver nuclei isolated from the same 12-day-old animal. Our results, shown in Fig. 2, revealed that [³H]UMP incorporation was approx. 3 times greater for liver nuclei than for myocardial-cell nuclei at this age. Also, [³H]UMP incorporation into nuclear RNA was strikingly increased by 100% at 60 min in myocardial-cell nuclei and to an even greater extent in liver nuclei (●, ■ symbols) in the presence of human placental RNAase inhibitor (1 unit/ μ l). The 3-fold difference in the absolute incorporation into nuclear RNA between liver and myocardial-cell nuclei remained present even with the addition of human placental RNAase inhibitor (●, ■ symbols).

RNA transcription in the heart from neonatal to adult development

To examine the relative rates of RNA synthesis and mitotic capacity in myocardial cells during postnatal

Fig. 2. Comparison of RNA transcription *in vitro* for ventricular myocardial-cell and rat liver-cell nuclei

Myocardial (■, □) and liver (●, ○) cell nuclei from 12-day-old tissue were incubated in parallel reaction mixtures at 26 °C in the presence (■, ●) or absence (□, ○) of human placental RNAase inhibitor. All points shown represent the mean and variance of four to six experiments. [³H]UMP incorporation was normalized to 1.75×10^6 nuclei at each time point.

Fig. 3. Myocardial-cell nuclear transcription with temporal development

Rat ventricular myocardial cell nuclei (3.5×10^7) from 5- (■), 10- (▲), 15- (●), 20- (○) and >100- (□) day-old rats were incubated in a 200 μ l reaction mixture in the presence of RNAase inhibitor at 26 °C for 60 min. Samples representing 1.75×10^6 nuclei were removed at pre-set time points and radioactivity incorporation into RNA was determined. All time points represent the mean and variance of four to six experiments.

Fig. 4. Hybridization analysis of myocardial-cell nuclear RNA with cDNA probe for the cardiac myosin-heavy-chain gene (pCMH21) and for the 'housekeeping' gene (pA50)

(a) Myocardial-cell nuclear RNA was obtained from rats 5 days (lanes 1 and 5), 10 days (lanes 2 and 6), 20 days (lanes 3 and 7) and >100 days of age (adult; lanes 4 and 8). Rat heart was fractionated (lanes 1-4, 5 μ g of RNA; lanes 5-8, 10 μ g of RNA), then transferred to nitrocellulose and subjected to hybridization. The cDNA probes pCMH21 (b) and pA50 (c) were radiolabelled as described in the Materials and methods section: (b) and (c) show hybridization patterns of the above probes to myocardial nuclear RNA.

development, myocardial-cell nuclei were isolated from 5-, 10-, 15-, 20- (representing decreasing DNA-synthesis capacity) and >100-day-old (representing mitotic quiescence) rat ventricular tissue and used for transcription *in vitro*. The rate of [³H]UMP incorporation, as shown in Fig. 3, exhibited a characteristic two-stage

Table 1. Quantitative analysis of Northern hybridization

Northern hybridization blots were performed as described in the Materials and methods section. A typical densitometric scan for pCMHC21 is shown. Scan A is representative of the densitometric analysis of the hybridization blot from 5 µg of myocardial-cell nuclear RNA. Scan B is representative of the hybridization blot from 10 µg of myocardial-cell nuclear RNA. Lighter-lined densitometric scans (1A, 1B) are of myocardial cell-nuclear RNA hybridization blots from 5-day-old rat heart. Darker-lined densitometric scans (2A, 2B) are of myocardial-cell nuclear RNA hybridization blots from 10-day-old rat heart. All integral values have been rounded to the next significant figure.

response for neonatal (5–20 days old) myocardial cell nuclei. The initial phase of rapid incorporation, which occurred from 0 to 15 min of transcription *in vitro*, was similar for all newborns studied. After this incubation period, a second phase of [³H]UMP incorporation was found to occur, with a detectable age-related decrease in absolute incorporation of radioactive material. Maximal incorporation of [³H]UMP occurred in 5-day-old myocardial-cell nuclei, with decreasing incorporation kinetics to 20 days of age. In direct contrast with the highly active neonatal myocardial-cell nuclei, adult (> 100 days of age) myocardial-cell nuclei exhibited low [³H]UMP incorporation into RNA. These results indicate that there is a greater than 10-fold decrease in RNA synthesis from 5 to 100 days of age.

Myosin-heavy-chain and 'housekeeping' gene transcription

In order to determine the temporal relationship between myocardial-cell nuclear transcription and specific gene expression during the development of the myocardial cell, the growth-related expression of the cardiac myosin-heavy-chain gene (α and β) and a general tissue 'housekeeping' gene was investigated. Hybridization with pA50 (Fig. 4c) indicated that the expression of the 'housekeeping' gene remained relatively stable throughout the lifespan investigated in the rat myocardial-cell nuclei. In contrast with this relatively constant extent of expression, hybridization with the cardiac myosin-heavy-chain cDNA (Fig. 4b) revealed that the detection of myosin expression was possible only before 20 days of age by using this methodology. As indicated in Fig. 3, there is at least a 10-fold decrease in expression between newborns and adults. The detection of the myosin-heavy-chain mRNA may be argued to be insensitive owing to RNA degradation; however, re-probing of the same samples with pA50 showed no detectable degradation. Therefore we believe that our results are representative of the steady-state nuclear contents of the

myosin-heavy-chain mRNA. In addition, we have analysed the Northern hybridization blots by scanning laser densitometry (Table 1). No detectable change in hybridization signal strength was found, indicating that degradation of nuclear RNA was not the causative factor leading to decreased detection of myosin heavy chain. These findings show that the regulation of RNA transcription associated with the temporal development of the myocardial cell is specific in nature, as reflected by the age-dependent decrease in certain gene products such as the myosin-heavy-chain gene in comparison with the age-independent expression of the 'housekeeping' gene.

DISCUSSION

The diversity between the myocardial and non-myocardial cells has been well documented (Alpert & Mulieri, 1986; Bugaisky & Zak, 1979; Chi *et al.*, 1975; Claycomb, 1973, 1979; Fine & Blitz, 1975; Liew *et al.*, 1983a,b; Morkin, 1983). Cellular division in the myocardial cells of the rat essentially ceases by week 3 of neonatal development and, after this period, the contractile components of the heart enlarge by the hypertrophy of pre-existing cells (Bugaisky & Zak, 1979; Claycomb, 1973, 1979). Our results indicate that a progressive decrease in the expression of nuclear RNA in rat ventricular myocardial-cell nuclei occurs from 5 days of age to adulthood, with a 10-fold decrease in [³H]UMP incorporation from a period of high mitotic activity to a period of relative mitotic quiescence. The mechanism(s) which account for the abrupt decrease in myocardial-cell nuclear transcription after the cessation of mitotic function (20 days of age) have yet to be elucidated. It has been previously reported that significant alterations in chromatin proteins (Liew *et al.*, 1983a), poly(ADP-ribose) synthetase activity (Jackowski & Kun, 1981, 1982) and DNA fragmentation (Jackowski & Kun, 1981)

are evident when 5-day-old and adult myocardial-cell nuclei were compared. These results suggest that multifactorial interactions of DNA-protein and protein-protein may play a role in transcriptional regulation during temporal development.

To determine the relative contribution of decreased RNA transcription during the period of overall cardiac enlargement, we have used a molecular approach to study the expression of specific constituent proteins in the myocardial cells by using two cDNA probes: pCMHC21 (the rat cardiac α myosin-heavy-chain gene with homologous α and β coding sequences) and pA50. Our results indicate that, for the housekeeping gene (pA50), no decrease in nuclear RNA expression occurred throughout the lifespan of the rat. However, in contrast with the 'housekeeping' gene, myosin-heavy-chain gene expression showed a marked decrease from 5 to 100 days of age in co-ordination with the decrease in RNA transcription and the previously reported decrease in DNA synthesis and mitotic activity (Claycomb, 1973, 1979).

The decrease in the requirements for cardiac myosin heavy chain owing to decreased mitotic activity and the relatively long turnover rate of 5 days would require lower extents of mRNA synthesis in order to maintain cellular growth. Our data concur with the findings of Zak (1986) and provide direct evidence that RNA transcription in the myocardial nucleus is progressively decreased during temporal development from the early newborn (a period of high mitotic activity) to the adult (a period of relative mitotic quiescence) in the myocardial cell.

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