A quantitative 'in-situ' hybridization method using computer-assisted image analysis

Validation and measurement of atrial-natriuretic-factor mRNA in the rat heart

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We have validated a quantitative 'in-situ' hybridization method and computer-assisted image analysis of autoradiographs as a technique for measuring atrial-natriuretic-factor (ANF) mRNA in tissue sections of rat heart by: (i) producing radioactive standards to calibrate the autoradiographs and (ii) assessing: (a) specificity (through RNAase A background subtraction, comparison of ANF mRNA and non-ANF mRNA probe binding to sections, Northern analysis and section-thickness titration curves); (b) sensitivity (by calculating the limit of detection for ventricular levels of ANF mRNA); (c) precision [inter-assay CV (coefficient of variation) less than 10%; intra-assay CV 6–7%]; and (d) accuracy. We have found with this technique that deoxycortone and saline treatment of rats elevates ANF mRNA to a larger extent in the ventricles than in the atria and that, in neonatal-rat hearts, ANF mRNA is elevated in all cardiac chambers relative to adult levels.

INTRODUCTION

Atrial natriuretic factor (ANF) is a circulating 28-amino-acid peptide present as a propeptide in storage granules in cardiac atria and, to a lesser extent, in the ventricles. It may be an important natriuretic factor controlling salt and water balance [1–6]. The measurement of specific ANF mRNA may demonstrate factors regulating ANF secretion through alteration of gene expression in the heart. Blotting methods have been used to determine the steady-state levels of these transcripts in various conditions [7–15]. The aim of our studies was to develop 'in-situ' hybridization as a quantitative technique allowing small changes in specific ANF mRNA levels to be measured and localized in single sections of the heart. This paper describes the validation of the procedure and, as part of this, a comparison of (1) hearts from vehicle- and deoxycortone/saline-treated rats, and (2) hearts from adult and neonatal rats.

MATERIALS AND METHODS

Rats

Adult male Porton-Wistar rats (Charles River) weighing 300–350 g were divided into two groups 4 weeks before removal of the hearts. The control animals were kept on water throughout, but the treatment group received only 2% (w/v) saline to drink and were administered one intraperitoneal 5 mg injection of deoxycortone pivalate (Ciba-Geigy) at the start of the salt-loading period. The neonatal pups of the same strain were used 3 days after birth.

Immediately after the rats were killed, the hearts were

rapidly removed and frozen on to specimen holders. Longitudinal frozen sections, 10 µm thick, were thaw-mounted and quickly dried on to gelatin-treated slides, which were then stored at –70 °C until used.

Probes

ANF-mRNA-directed probes. (a) A 42-mer synthetic oligodeoxyribonucleotide probe (G + C content = 64%) complementary to the mRNA coding for ANF-(103–116)-peptide [16] was purified by denaturing PAGE and passage through a mini DEAE-cellulose anion-exchange column.

The ³⁵S-labelled dCMP 3' end-tailed ANF probe was produced by using deoxycytidine 5'-[α-³⁵S]thio]triphosphate (dCTP[³⁵S][S]) (> 600 Ci/mmol; Amersham International and New England Nuclear) and terminal deoxynucleotidyltransferase (Pharmacia). The incorporation rate and specific radioactivity were determined by using DE 81 filter paper (Whatman) or separation through a Sephadex G-50 minicolumn. Typical specific radioactivities were (5–30) × 10¹⁷ d.p.m./mol.

The ANF mRNA oligonucleotide probe was also labelled to a high specific radioactivity with a single [³⁵S]thiophosphate at the 5' end or a single [³⁵S]nucleotide at the 3' end using adenosine 5'-[γ-³⁵S]thio]triphosphate (ATP[³⁵S][S]) (> 1000 Ci/mmol; Amersham and New England Nuclear) and phage-T₄ polynucleotidyl kinase (Pharmacia) or dideoxyadenosine 5'-[α-³⁵S]thio]triphosphate (ddATP[³⁵S][S]) (> 1000 Ci/mmol, NEN) and terminal transferase respectively. Good incorporation rates were obtained, producing probes of high specific radioactivities [(3–30) × 10¹⁷ d.p.m./mol].

(b) The plasmid, phAF48, was a gift from Professor S.

Abbreviations used: ANF, atrial natriuretic factor; CI, confidence interval; POD, photographic optical density; CV, coefficient of variation; SSC, standard saline citrate (0.15 M-NaCl/0.015 M-sodium citrate); LA, left atrium; RA, right atrium; PAGE, polyacrylamide-gel electrophoresis; dCTP[S], deoxycytidine 5'-[α-thio]triphosphate; ATP[S], adenosine 5'-[γ-thio]triphosphate; ddATP[S], dideoxyadenosine 5'-[α-thio]triphosphate.

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Nakanishi (Institute of Immunology, Kyoto University Faculty of Medicine, Kyoto, Japan; see [17]). The 581-bp *SacI*–*PstI* restriction fragment containing the complete ANF coding sequence was labelled with dCTP[³⁵S][S] using the random hexanucleotide method (Multiprime labelling kit; Amersham International) to a specific radioactivity of 5×10^8 d.p.m./ μ g (equivalent to $\sim 10^{20}$ d.p.m./mol).

The hybridization buffer containing the labelled restriction fragment was boiled for 10 min and rapidly cooled on ice before use.

'Non-specific' binding probes. These consisted of (a) a 50-mer oligodeoxynucleotide (G + C content = 44 %) complementary to a sequence approx. 200 bases upstream of the postulated initiation site of ANF mRNA transcription [16] and (b) a 45-mer 'sense' oligodeoxynucleotide (G + C content = 64 %) identical with a region of the mRNA coding for rat insulin-like growth factor II.

These synthetic oligonucleotides were purified and 3'-end-tailed with terminal transferase.

Northern analysis

Total cellular RNA was extracted from adult rat heart by a modified guanidinium isothiocyanate method [18] and stored in ethanol at -20°C .

After denaturation and separation on a 1%-agarose/formaldehyde gel, the RNA was transferred on to Hybond N membrane (Amersham International) using a Vacugene blotting system (LKB) as directed by the manufacturers.

The membrane was pre-hybridized for 1 h at 37°C in a solution of final composition: 0.9 M-NaCl, 50 mM-sodium phosphate, pH 7.7, 5 mM- Na_2EDTA , 50 % deionized formamide, 5 $\times$ Denhardt's solution (500 mg of polyvinylpyrrolidone, 500 mg of bovine serum albumin and 500 mg of Ficoll 400 in 500 ml of distilled water) and 0.5 % SDS. Overnight hybridization at 37°C was performed in the same solution containing 40 pmol of the probe labelled with ATP[³⁵S][S]/20 ml. The membrane was then washed with increasing stringency down to $0.1 \times \text{SSC}/0.1\%$ SDS/14 mM- β -mercaptoethanol. After being allowed to dry, it was apposed to autoradiography film (Hyperfilm β max; Amersham International).

'In-situ' hybridization

After removal from the -70°C freezer, the sections were fixed for 5 min in 50 mM-phosphate-buffered saline (0.1 M-sodium phosphate/0.15 M-NaCl, pH 7.4) containing 4 % formaldehyde, followed by two washes in $2 \times \text{SSC}$, pH 7.0. They were prehybridized for 1 h at 37°C with a buffer (previously boiled for 10 min and rapidly cooled on ice) containing (final concentrations) 50 % deionized formamide, $2 \times \text{SSC}$, 5 $\times$ Denhardt's solution and sheared herring sperm DNA (250 μ g/ml). The sections were then washed briefly in $2 \times \text{SSC}$ and air-dried before the application of the same solution to which had been added dextran sulphate (final concn. 5 %), β -mercaptoethanol (final concn. 40 mM) and the labelled probe. In all experiments, except those on concentration effects, 500 fmol of labelled probe was applied to each section at a concentration of 10 fmol/ μ l.

After overnight hybridization at 37°C , sections were washed with increasing stringency (down to $0.1 \times \text{SSC} + 14$ mM- β -mercaptoethanol at 37°C for the oligonucleotide or 50°C for the cDNA probe), progressively

dehydrated in ethanol solutions diluted in 300 mM-ammonium acetate, and then air-dried.

RNAase A treatment of sections

Formaldehyde-fixed sections were pretreated with RNAase A (Sigma), previously boiled to destroy DNAase activity, at a concentration of 100 μ g/ml in 0.5 M-NaCl/10 mM-Tris/1 mM-EDTA, pH 8.0, for 30 min at 37°C and then incubated for a further 30 min at 37°C in the buffer alone. The sections were re-fixed for 5 min in 4 % buffered formaldehyde and rinsed in $2 \times \text{SSC}$ twice before application of pre-hybridization and hybridization solutions.

Computer-assisted image analysis of autoradiograms

The dry sections were exposed to autoradiography film (Kodak X-Omat XS1 or Hyperfilm β max; Amersham International), and the autoradiograms were analysed by using a Quantimet 920 image-analysis system (Cambridge Instruments, Cambridge, U.K.) by adapting a computer program developed for quantitative receptor autoradiography [19].

The autoradiograms were quantified by comparison with a calibrated ³⁵S radioactive scale co-exposed with the hybridized sections [20]. The integrated diffuse photographic optical density (POD) and area were measured for each standard to calculate radioactivity in d.p.m./mm², and a calibration curve (natural logarithm of the POD density versus radioactivity) was generated for each film (an example of which is shown in Fig. 1).

The POD of discrete regions in the atria and ventricles were measured and converted into the amount of radioactivity bound to the tissue section by interpolation from the standard curve. The image of the non-specific binding of the probe to an adjacent section treated with RNAase A was digitally subtracted from the 'total' image to obtain the amount of probe bound to RNAase-digestible sites ('specific' hybridization).

Fig. 1. Standard curve of POD versus radioactivity

An example of a plot of ln(POD) versus ln[radioactivity (d.p.m./mm²)] for 10 μ m-thick tissue paste standards containing dCTP[³⁵S][S]. There is a linear relationship between these parameters, with a slope of 0.812 and an intercept of -5.8 ($r = 0.99$; $P < 0.001$). Each value represents the mean of three determinations. S.E.M. values were less than 3 % of the mean.

All results have been converted into units (amol/mm²) of 'specifically' bound probe by using the equation:

$$\text{Probe bound (amol/mm}^2\text{)} = \text{d.p.m./mm}^2 \times \text{SR}^{-1} \quad (1)$$

where SR is the specific radioactivity of the probe (d.p.m./amol) at the midpoint of exposure of the sections to the autoradiographic film.

Statistical analysis

Data were shown to have a high correlation with normality by using the Shapiro & Francia probability plot correlation test for non-normality [21]. Results were analysed using the unpaired two-tailed Student's *t* test. Transformed data were compared with the Mann-Whitney U-test.

The results of the effect of increasing section thickness and probe concentration were subjected to linear-regression analysis. Testing for significance revealed that linearity was lost at the highest concentration used. The data were truncated one point at a time until a linear model could be accepted. Linearity was confirmed by examining the plots of the residuals. 95 % confidence intervals (CI) for the slope and intercept were calculated.

RESULTS AND DISCUSSION

The 'in-situ' hybridization protocol used in the present study has been based on the extensive work performed by others on the factors required for optimal results (e.g. see [25–28]), but previous studies have quantified probe binding by counting the number of silver grains [26,29,30]. However, numerous factors affect the response of the emulsion to radiation and, as a result, measurement of grain density is difficult to make with precision [29]. These factors can be controlled for, or avoided, by co-exposing the radiolabelled sections with a set of calibrated standards that closely resembles the experimental source. As single-cell resolution was not required, we have measured POD in film-based emulsions by computer-assisted image analysis. The POD is then converted into absolute amounts of radioactivity bound by using a standard curve, unlike other studies which have expressed results in relative quantities of probe binding [26,31,32].

Specificity

We have screened our ANF mRNA probe and found that there is no significant complementarity of this probe to other nucleic acid sequences in the EMBL and GenBank nucleic acid databases.

After 'in-situ' hybridization, the binding of the 'non-specific' probes was very low and showed no differentiation between atrium and ventricle and between RNAase-A-treated and untreated sections. This indicates that cardiac tissue does not possess unusual DNA binding properties. The similar atrium/ventricle binding ratios of the 42-mer ANF mRNA probe and the *SacI*–*PstI* restriction fragment were abolished, with a large reduction of total binding, by RNAase A pretreatment of the sections. However, the larger cDNA always produced fainter autoradiographic images [25,35]. Subtraction of the images from the RNAase A-treated sections eliminates non-specific binding to non-RNA molecules. At the concentration employed (100 µg/ml), RNAase A cleaves single-stranded RNA molecules, including the region in the ANF mRNA complementary to our probe

Fig. 2. Northern analysis of cardiac RNA using the ANF mRNA probe

Identification of ANF mRNA by Northern-blot hybridization analysis of total adult-rat atrial (a) and ventricular (v) RNA. Total RNA (20 µg) was electrophoresed in a 1% agarose/formaldehyde gel for 150 V·h. The RNA was transferred to Hybond N nylon membrane and hybridized overnight with the [³²S]thio-phosphate-labelled ANF oligonucleotide. The resulting autoradiogram of atrial RNA shows a major band of radioactivity (large arrow) corresponding to the expected position of cardiac ANF mRNA [relative to RNA size markers (BCL); positions of 28 S and 18 S rRNA are shown]. The fainter bands are degradation products and are not a consistent finding. No bands were seen in the ventricular sample.

[33,34], even though double-stranded RNA is relatively resistant [22].

Northern analysis of total cellular RNA from adult rat heart using the 42-mer ANF mRNA probe is shown in Fig. 2. There is a single major band of radioactivity corresponding to the expected position of cardiac ANF mRNA [7,9,17]. The absence of banding at the positions of rRNA and tRNA suggests that the probe does not interact with these within the tissue section. However, tRNA (and 5 S rRNA) may remain solubilized during acetate/ethanol precipitation [36]. Nevertheless, these polynucleotides are predominantly double-stranded, and non-specific hybridization to them should be low.

The effect of increasing cryostat section thickness on 'specific' hybridization is shown in Fig. 3. There is a linear relationship between section thickness over the range 5–30 µm and the amount hybridized for both atria and ventricles. The slope (7.85; CI 9.58–6.22) of the titration curve for control adult atria is significantly steeper (*P* < 0.001) than that of the binding to ventricular tissue (0.195; CI 0.228–0.16), with no significant

Fig. 3. Effect of section thickness on ANF mRNA probe binding

The effect of increasing cryostat section thickness on the amount of the 5'-³⁵S-labelled ANF mRNA probe bound to adult-rat atria and ventricles. There was a linear relationship between the section thickness and the amount of labelled oligonucleotide hybridized, with a significant difference ($P < 0.001$) between the slope of the atrial line (slope = 7.85, 95% CI 9.48–6.22) and that of the ventricular one (slope = 0.195; 95% CI 0.16–0.228). There was no significant difference between the y intercepts of the two lines (atrium: -26.8 ; 95% CI 0.2 to -53.8 ; ventricle: -1.58 ; 95% CI -1.03 to -2.13). Each value represents the mean of four determinations ($\pm$ S.E.M.). a, atrial titration line; v, ventricular titration line.

difference in y intercepts of the two lines (atria: -26.8 , 95% CI 0.2 to -53.8 ; ventricles: -1.58 , 95% CI -1.03 to -2.13). This suggests that (a) increasing section thickness raises the total amount of RNA/unit volume, (b) in the ventricles, ANF mRNA forms a smaller proportion of total RNA than in the atria, and (c) the probe is only hybridizing with this specific mRNA [22]. The ratio of the atrial to ventricular slopes is similar to the differences between atrial and ventricular mRNA measured (in arbitrary units) by blotting methods [8,9,13].

Sensitivity

Effective probe excess was achieved by varying the concentrations of the ANF mRNA probe used. Fig. 4 shows the results using the same 42-mer ANF mRNA probe labelled by two different methods: 'a' at the 5' end with ATP[³⁵S][S], and 'b' at the 3' position with ddATP[³⁵S][S]. There was a linear relationship between probe concentration (0.5–8 fmol/ μ l) and the amount of oligonucleotide 'specifically' hybridized to control adult atrial sections, which reached a plateau above 8 fmol/ μ l, unlike the situation expected for unsaturable, non-specific, binding.

The results in Figs. 3 and 4 are equivalent to titration curves of the proportion of specific ANF mRNA in total RNA pools; a doubling of either the probe concentration or the section thickness doubles the amount of probe

Fig. 4. Effect of probe concentration on specific hybridization to rat atrial sections

The effect of increasing the concentration of 2-³⁵S-labelled ANF mRNA probes (a was labelled with [³⁵S]thio-phosphate at the 5' end; b was labelled at the 3' position with ddATP[³⁵S][S] using terminal transferase). When the data for the highest concentration were omitted, for both probes there was a linear correlation between specific probe hybridization and probe concentration, without a significant difference between the slope for probe a (7.34; 95% CI 6.22–9.48) and that for probe b (6.48; 95% CI 6.12–6.84). The amount of probe hybridized reached a plateau above a concentration of 8 fmol/ μ l when applied in a volume of 50 μ l. Each value represents the mean of four determinations ($\pm$ S.E.M.).

bound. This suggests that all the sites available for hybridization are being detected. The slopes of these lines can be used to determine transcript prevalence/cell [22] using the equation:

$$T = (N/b) \cdot m \cdot S \quad (2)$$

where T is the number of transcripts/cell, N is Avogadro's number, b the specific radioactivity of the probe, m the slope of the titration curve in units of RNAase-digestible binding per mass unit of cellular RNA and S the mass of RNA/cardiac cell (approx. 2×10^{-5} μ g/cell). From eqns. (1) and (2) we have calculated that the limit of detection is less than five copies of ANF mRNA/cell, which is comparable with that achieved with solution hybridization (< 5 transcripts/cell). These calculations indicate that our technique is considerably more sensitive than blotting methods, which have either been unable to detect ventricular ANF mRNA [7,11,12,17] or require pooled extracts from several hearts in order to do so [8]. In addition, there is no disruption of anatomical integrity, and we avoid the need for the large cDNAs required for solution hybridization or ³²P-labelled probes which give less precise tissue localization (D. J. Nunez & P. C. Emson, unpublished work).

Precision

The intra-assay coefficient of variation (CV) for two sets of serial atrial sections was 5.7 and 6.7%, and, for the corresponding ventricular sections, 5.7 and 6.6%.

Fig. 5. Autoradiographical localization of the ANF probe hybridization

Examples of the localization of the specific binding of the ddAMP[³⁵S][S] labelled ANF mRNA probe to sections from a single normal adult rat heart. After hybridization, sections were washed, dried and apposed to radiation-sensitive film. The effect of increasing probe concentration is shown in (a) (0.5 fmol/μl) and (c) (11.8 fmol/μl). (b) Demonstrates the image obtained from an RNAase-treated section hybridized at 0.5 fmol/μl (N.B.: photographed at greater sensitivity to permit visualization). The images in (d)–(f) were obtained by hybridization to sections of increasing thickness (5 μm, 10 μm and 25 μm respectively). The POD of the images increases with increasing probe section thickness and concentration (see Figs. 3 and 4 for pooled data).

The interassay CV was less than 10%. These compare favourably with those of other assay methods, such as radioimmunoassay, and are critical to dispelling the current view of the value of 'in-situ' hybridization only as a qualitative technique.

Accuracy

The lack of a fully validated alternative method for measuring specific ANF mRNA has made it difficult for us to ascertain the accuracy of our results. Conventional blotting and solution hybridization techniques, as methods for quantifying specific mRNA, have an accuracy which is not easily quantifiable, owing to unknown and perhaps variable factors such as extraction and transfer losses and hybridization efficiency. We are unable to assess our efficiency of hybridization or quantify the loss of mRNA during section preparation, storage, fixation and hybridization, but the latter is probably small [27,28], and this contributes to the low variation of our measurements. We have not directly compared results obtained with our technique and those produced by these other methods.

Recently we have found internal consistency between the results with two oligonucleotide probes complementary to different regions of the ANF mRNA (D. J. Nunez, unpublished work). In addition, the similarity of our results concerning changes in ANF mRNA expres-

sion under various circumstances with those reported using pooled cardiac mRNA is also reassuring.

ANF mRNA

Fig. 5 shows typical examples of the autoradiograms obtained with the 42-mer ANF probe after hybridization to normal adult-rat heart. The POD of the images increases with increasing probe concentration (Figs. 5a and 5c) and section thickness (Figs. 5d–5f). Binding to an RNAase-treated section is very low, without a difference between atria and ventricles (Fig. 5b). The effects of mineralocorticoid/saline treatment on the autoradiographical images obtained after hybridization with the ANF mRNA probe are shown in Fig. 6. The adult control atria (a) bind the highest amount of probe with lower binding in the ventricles (v). In the mineralocorticoid-treated, saline-loaded rat, the levels in the atria and ventricles are elevated compared with controls.

The data on ANF mRNA levels (Fig. 7) in adult control (white bars) and saline/deoxycortone-treated rats (hatched bars) show that there is no significant difference between ANF mRNA probe hybridization to left (LA) and right atria (RA) in the two groups [controls: LA, 27.8 ± 3.01 (mean $\pm$ S.E.M.); RA, 28.62 ± 3.13 ; treated: LA, 47.82 ± 3.46 ; RA, 49.4 ± 6.9 amol/mm²]. Lattion *et al.* [8] reported similar results, but others [12,14] have found differences between ANF mRNA prevalence

Fig. 6. Effect of mineralocorticoid/saline treatment on ANF probe hybridization

In the control rat heart (a), the atria (a) bind the highest amount of probe with detectable, but lower, binding in the ventricles (v). In the mineralocorticoid-treated, saline-loaded rat (b), levels in the atria and ventricles are elevated compared with controls.

Fig. 7. Binding of the ANF probe to adult-rat hearts

Specific hybridization of the 3'-end-labelled ANF mRNA probe to adult control (white bars) and saline/deoxycortone treated rats (hatched bars). In the control animals there was no significant difference between left (LA) and right atrial (RA). The levels of ANF mRNA probe binding in atria and ventricles were significantly elevated in the saline/mineralocorticoid-treated rats ($P < 0.05$; one-way analysis of variance, followed by t test). Each value represents the mean of 12 determinations ($\pm$ S.E.M.).

in RA and LA. Atrial binding in the controls is approx. 70 times greater than ventricular (control ventricles: 0.41 ± 0.08 , $P < 0.0001$), as has been reported previously [8,13,14]. The levels of hybridization to the atria and ventricles of the treated rats were significantly elevated ($P < 0.05$) when compared with those of control animals, but the ventricular binding showed a greater relative increase (treated ventricles: 1.00 ± 0.15).

In agreement with Bloch *et al.* [23] and Wei *et al.* [24], in the neonatal-rat hearts there was no significant difference between probe hybridization to LA and RA (LA, 77.13 ± 6.91 ; RA, 77.04 ± 8.29 amol/mm²), but significantly lower levels were detected in the ventricles (16.42 ± 3.16 amol/mm²; $P < 0.01$). However, in our study at the 3-day-neonatal stage, all cardiac tissues exhibited levels of probe hybridization greater than those in the control adults.

In conclusion, we have shown that 'in-situ' hybridization and computer-assisted image analysis of autoradiographs can provide quantitative information on specific mRNA levels in defined areas of tissue sections. Although we have focused on the measurement of ANF mRNA in the rat heart, the technique should be useful in situations when preservation of anatomical relationships is important in small areas of tissue, samples are small (e.g. human biopsy specimens) and transcripts are present at low prevalence.

We are grateful to Mr. A. Northrop and Dr. R. S. Gilmour for the synthesis of the oligonucleotides and helpful discussions and to Professor S. Nakanishi for the gift of plasmid pAF48. D. J. N. thanks the Trustees of Addenbrooke's Hospital and the East Anglian Regional Health Authority for financial support, and Squibb for a Squibb Cardiovascular Fellowship.

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Received 7 November 1988/21 March 1989; accepted 4 April 1989