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The application of aqueous two-phase partition to the study of chick limb mesenchymal diversification

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SUMMARY

A technique which identifies cells differing in surface character, aqueous two-phase partition using thin-layer countercurrent distribution (TLCCD), has been used to study differentiation and pattern formation in the developing chick limb bud.

The TLCCD profiles of cell populations, derived from various regions of morphologically undifferentiated mesenchyme from three different stages of limb development, have been compared.

At no stage, or location, has the population been found to be homogeneous. Cells from progress zones and more proximal regions could all be resolved into several populations. The populations from progress zones at three different developmental stages were qualitatively similar but differed in the proportions of cells in each. The most striking differences in cell populations were those obtained from the most proximal region of the limb, closest to the flank, which represents the developmentally most advanced region.

INTRODUCTION

The chick limb bud has been widely used as an experimental model for the study of pattern formation in developmental fields. Its value lies in the fact that experimental intervention is possible from the earliest stages until the point at which the pattern is complete. The pattern of chondrogenic elements, as revealed in whole mounts prepared some days later, provides a convenient end point against which the effects of the manipulation can be analysed. This type of study has provided much information as to the role of positional information in skeletal pattern formation, leading to the formulation of a number of models based on this principle (for review see Hinchliffe & Johnson, 1980).

It has been suggested that the establishment of the limb pattern, at a cellular level, involves the diversification of a pluripotent population, yielding a heterogeneous population of chondrogenic and fibrogenic precursor cells in an organized spatial array (Archer, Cottrill & Rooney, 1984; Solursh, 1984; Cottrill, Archer,

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Hornbruch & Wolpert, 1985). This heterogeneity is increased further, and possibly influenced by the influx of the somite-derived myoblast precursor cells (Christ, Jacob & Jacob, 1974; Chevallier, Kieny & Mauger, 1976). This hypothesis is based on the interpretation of *in vitro* experiments of limb bud mesenchyme micromass culture morphology and differentiation.

The aim of the work presented here was to determine whether such heterogeneity exists, and can be detected *in vivo*. The technique of repetitive aqueous two-phase partition (cell surface chromatography) (Albertsson, 1971; Sharpe, 1984), which identifies and separates cells having different surface properties was used. It was assumed that differences between cells at early developmental stages will be small and are likely to involve surface properties. On the basis of these assumptions, cell surface chromatography would appear to be an ideal method, since it allows small differences in surface character to be detected (Walter, 1977). The technique depends on the ability of mixtures of aqueous solutions of dextran and poly (ethylene glycol) to separate, on standing, into two phases, the upper phase being poly (ethylene glycol)-rich and the lower phase dextran-rich (Albertsson, 1971). When mixed with such a phase system, cells with different surface properties have different affinities for the two phases and therefore separate. It is usually necessary to repeat such cell partitioning many times in a repetitive countercurrent fashion with fresh phases to obtain good separation (cell surface chromatography) (Sharpe, 1984).

MATERIALS AND METHODS

Cell suspensions

Festival hens' eggs from a local flock (Mytholmroyd Hatchery, Hebden Bridge, W. Yorkshire) were incubated at 38 ± 1°C. The embryos were then transferred to a Petri dish containing calcium- and magnesium-free saline (CMF) supplemented to 10% newborn calf serum (Gibco), pH 7·2, and staged according to Hamburger & Hamilton (1951) under a Zeiss Stereomicroscope (Zeiss, FRG). The wings were then removed at the flank using tungsten needles, washed in CMF and transferred to fresh CMF in a second Petri dish. Further dissection was carried out under the same optics, with a micrometer eyepiece graticule, using a Graefe cataract knife (Weiss, London) and tungsten needles. The following proximodistal limb regions were used: stage-21 progress zone and subprogress zone, stage-22+ progress zone, subprogress zone and proximal, and stage-25 progress zone. The proximodistal limit of the progress zone was derived from a consideration of morphological, biochemical and pattern regulation data (Cottrill *et al.* 1985). The limb fragments were transferred to a cold solution of 2% trypsin in CMF and incubated for 15 min at 4°C. Subsequently they were washed in serum-supplemented CMF and the ectoderm dissected free from the mesenchyme using tungsten needles under the stereomicroscope. The isolated mesenchyme pieces were then dissociated in CMF by vortex, the cells washed in the same solution and the suspension filtered through a double layer of Nitex mesh (pore size 20 μm). For partition, the cells (approximately $5{\cdot}0\times10^6$), were washed in top phase and resuspended in 0·9 ml of this solution immediately prior to being loaded into the first chamber of the rotor (see below).

Phase partition

A low potential phase system, composed of 5·2% (w/w) dextran T500 (Pharmacia, batch No. HD26066), 5·2% (w/w) poly[ethylene glycol] 4000 (BDH Chemicals, UK), 0·05 mol kg^{-1} NaCl, 0·01 mol kg^{-1} disodium phosphate buffer, pH 7·0, 0·02% (w/w) EDTA, 5% foetal calf serum

(Gibco) and 5% (w/w) sucrose, was employed throughout. The phases were prepared in bulk and allowed to equilibrate for 5 days at 4°C, after which time the top and bottom phases were separated and stored frozen until used.

Thin-layer countercurrent distribution was carried out at 4°C on a Bioshef Mark 1 TLCCD apparatus with a 60-chamber rotor, each of the 60 transfers comprising a mixing time of 30 s and a settling time of 9 min. Cells were unloaded from the rotor in phosphate-buffered saline and aliquots from alternate fractions counted on a Coulter counter.

Analysis of TLCCD profiles

Since each profile can be considered the sum of a number of Poisson distributions for each homogeneous subpopulation (with respect to surface properties) of the whole, the number and size of each component population can be estimated mathematically. This was carried out using a computer programme originally devised Blomquist & Wold (1974) and extensively modified by Dr G. C. Ford of the Department of Biochemistry, University of Sheffield, with which the number and size of Poisson distributions producing the best fit for the observed profile can be calculated. The application of such a programme is only possible where the rotor used has been shown, as here (Sharpe, unpublished), to be capable of producing a theoretical distribution for a homogeneous population of cells.

The data for each population therefore comprise the actual TLCCD profile and one constructed by the programme, combining the smallest number of Poisson distributions to produce the best fit. The accuracy of the latter can be estimated from a comparison of the areas under the actual profile and that constructed. This was carried out for the profiles presented here, using an IBAS I Image Analysis System (Kontron, FRG), a mean error of 5·14% being found.

Cell viability, recovery and morphology

A sample of cells was removed from the peak fraction of each TLCCD population obtained and their viability assayed by the trypan-blue-exclusion test. Typical viabilities obtained ranged between 75 and 90% with no consistent differences observed with cells from any particular peak. Total cell recovery was estimated by calculating the total cell number in all fractions from the Coulter counts. Recoveries obtained were around 75% of total cells added.

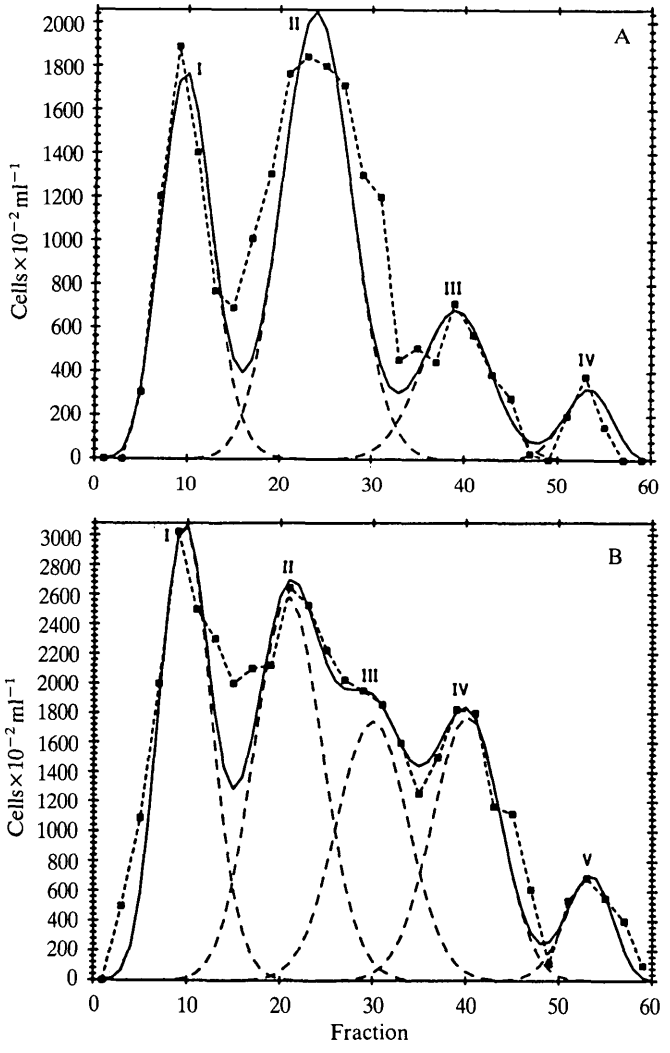
The morphology of cells in the different populations was not examined in any detail. Light microscopy observations showed that the majority of the cells were single (i.e. not clumped) but at this level no obvious morphological differences were visible.

RESULTS

The TLCCD profiles and their computer analyses are illustrated in Figs 1, 2 and 3. The populations analysed provide information as to spatial differences of cell surface properties within a single stage limb bud and temporal differences of these properties within similar regions from different stage limb buds. All of the profiles presented below have been found to be qualitatively repeatable, though some quantitative differences in the size of the subpopulations revealed by the computer analysis were obtained, an example is shown in Fig. 4 which is the profile obtained in a repeat of the experiment in Fig. 2A.

Progress zone profiles

In the three stages investigated the progress zone has been defined as that region of mesenchyme lying within 350 μm of the AER. This provides a comparison of the surface properties of an analogous population from different stage limb buds.



Figs 1–3. TLCCD profiles of cells dissociated from different limb-bud regions after 59 partition steps. □, Experimental points; ----, individual cell populations; ———, sum of individual cell populations.

Fig. 1 (A) Stage-21 progress zone; (B) stage-21 subprogress zone.

None of the TLCCD profiles shows a theoretical distribution for a single homogeneous population. The progress zone profiles of all three stages show four cell populations. Stage-22+ and -25 progress zones have similar profiles where all four populations have peaks around the same fraction numbers. The proportion of cells in each population is, however, different for each progress zone. The stage-21 progress zone profile appears visibly different from both stages 22+ and 25. However, populations I and III of stage 21 have the same position as populations I and IV of stages 22+ and 25. Population IV of stages 22+ and 25, however, represents only a minor cell populations whereas population III of stage 21 constitutes around 15% of the total cell number. Population II, stage 21 occupies

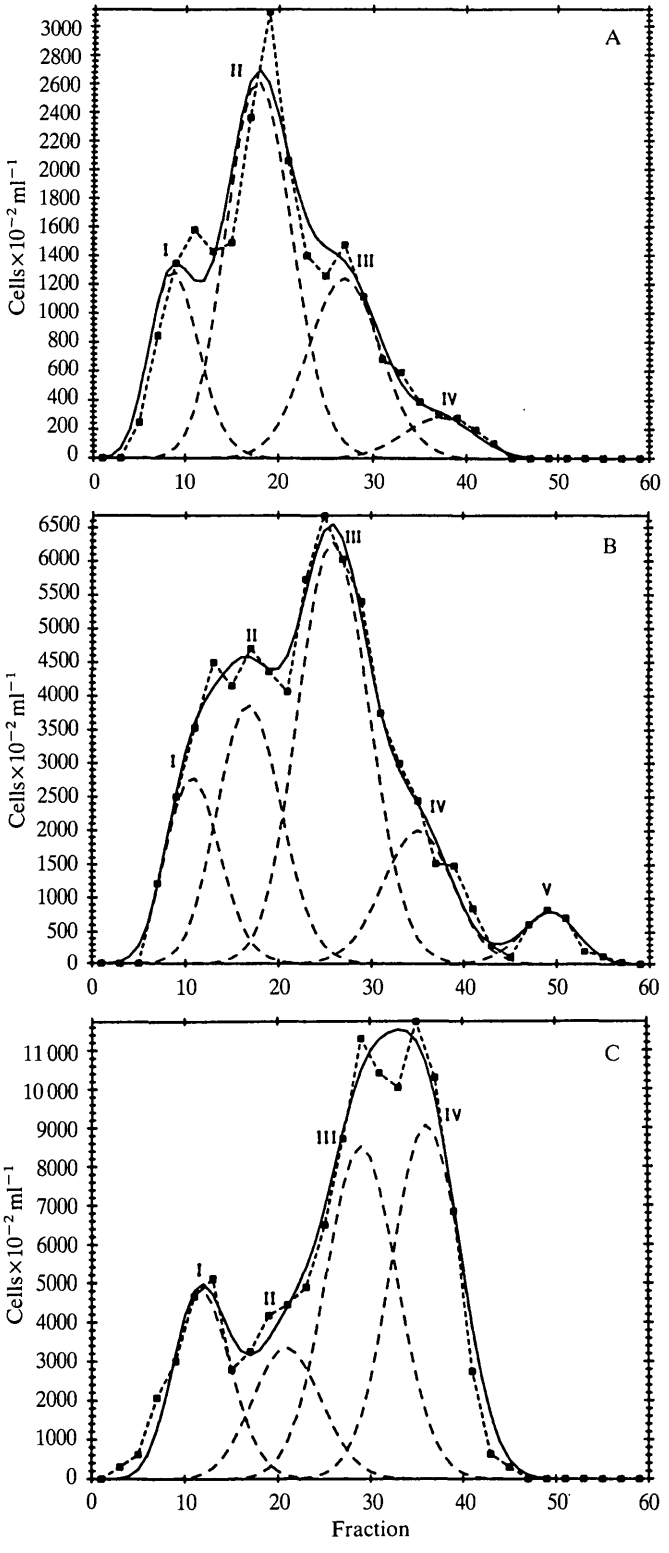


Fig. 2. (A) Stage-22+ progress zone; (B) stage-22+ subprogress zone; (C) Stage-22+ proximal region.

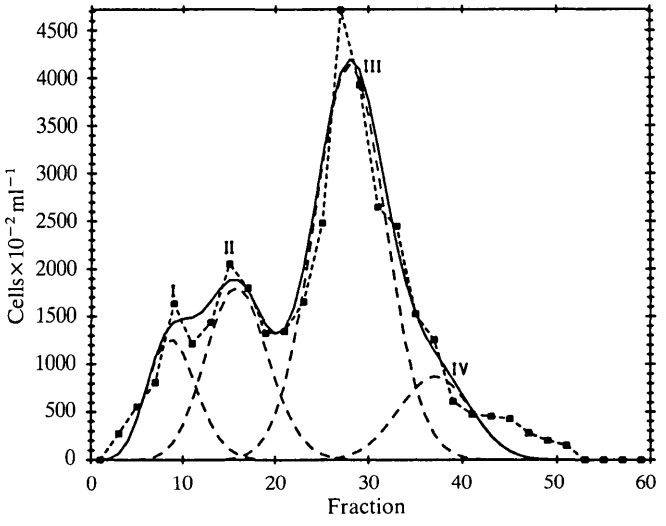


Fig. 3. Stage-25 progress zone.

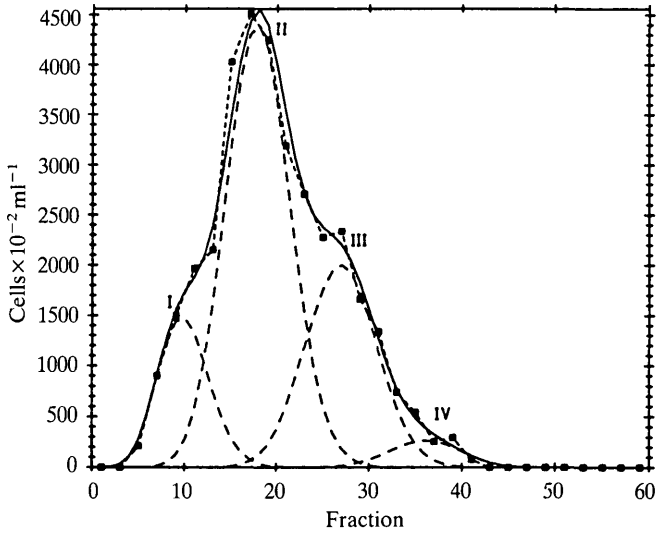


Fig. 4. Stage-22+ progress zone (repeat).

an intermediate position between populations II and III of stages 22+ and 25 and stage 21 has a unique small population at the extreme right of the profie (IV).

Subprogress zone profiles

A comparison of the surface properties of the progress zone and the corresponding subprogress zone mesenchymal cells furnishes information as to the spatial differences of these properties within the single developmental stage limb bud.

Stage 21

The subprogress zone cells at this stage can be resolved into five subpopulations equally spaced across the chambers. Three of these populations are coincident with those of the progress zone, though their relative heights differ. However, the second population of the progress zone is replaced by two populations in the subprogress zone.

Stage 22+

The subprogress zone cells can be resolved into five subpopulations, the first four of which are coincident with those of the progress zone at this stage, with the additional populations lying to the extreme right (Fig. 2A,B).

A further region from the stage-22+ limb was analysed by TLCCD. This was derived from that portion of the limb remaining after the removal of the progress- and subprogress-zone mesenchyme and was designated 'proximal' mesenchyme. The TLCCD profile is clearly skewed to the right-hand side of both the progress- and subprogress-zone profiles at this stage and consists of four populations (Fig. 2C). This profile appears to bear little resemblance to any of the others either in overall distribution or distribution of individual subpopulations. There appear to be no populations in the proximal profile comparable to the small one located around fraction 50 of the subprogress-zone profile.

DISCUSSION

It has been suggested previously from micromass culture observations that the progress zone throughout its existence comprises a uniform population of pluripotent cells (with respect to the fibrogenic and chondrogenic phenotypes) upon which the mechanisms of pattern formation act to restrict the expression of the chondrogenic phenotype to the prospective skeletal areas of the limb (Archer *et al.* 1984; Cottrill *et al.* 1985). This sequence of events in skeletal pattern formation has also been proposed by Solursh (1984). However, it is clear from the results presented here that the progress zone cannot be considered as homogeneous, at least with respect to the cell surface properties of its constituent cells.

In both situations where the progress zone and corresponding subprogress zone cells have been compared, it is apparent that there are no major differences in the number and relative positions of the populations. The subprogress zones are, however, slightly more heterogeneous than the corresponding progress zone, having one more detectable cell population. There are also major differences in the relative proportions (cell number) of each population. The progress zone model predicts that the pattern of skeletal and soft tissue elements becomes fixed as the cells leave the influence of the apical ectodermal ridge due to the pressure of more distal cell division. The results presented here illustrate that if positional value is acquired at this time, it is probably not expressed as a wide range of cell surface differences. The additional cell population seen in the subprogress zone profiles may reflect the presence of somite-derived myoblast precursor cells since

these would be largely absent in the progress zone at these stages (Rutz & Hauscka, 1982). Indeed it has been recently shown that any differences observed between progress zone and subprogress zone mesenchyme micromass cultures can be explained by the presence of myoblast precursor cells (Cottrill *et al.* 1985).

One interesting observation is that the additional population in stage-2 subprogress zone (III) is almost coincident with population III from stage-22+ proximal region. The stage-22+ proximal region largely originates from the stage-21 subprogress zone region. The most striking differences observed are also seen in cells from stage-22+ proximal region. Here the overall shape of the profiles bears little resemblance to the profiles from other regions. Significantly it is this region that represents the most developmentally advanced of those studied.

Clearly at this stage little can be said concerning the phenotype of any of the different populations. It is hoped that cells can be isolated from each population and their morphology studied, particularly in micromass culture, to reveal any differences in phenotype. One possibility that should not be excluded at this time is that some of the populations may represent cells of the same phenotype at different stages of the cell cycles.

Cells from each population do have different surface properties, since this was the basis of the separation procedure. However, at the present time, cell surface chromatography provides little information concerning the nature of the surface differences detected. The phase system used, being of a low potential type, is relatively insensitive to differences in surface charge. The surface differences detected in such systems are generally referred to as differences in hydrophobicity. Thus cell populations to the left (lower fraction numbers) can be considered to have a less hydrophobic surface than cell populations to the right, in higher fraction numbers.

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