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Heart C-Protein Is Transiently Expressed during Skeletal Muscle Development in the Embryo, but Persists in Cultured Myogenic Cells

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The expression of cardiac and white skeletal C-protein isoforms was analyzed in developing chicken embryos and in primary skeletal muscle cell cultures by immunoblot and immunofluorescence staining using polyclonal antibodies specific for both of the two different proteins. In the embryo, cardiac C-protein was detected in the developing heart from very early stages through adulthood. In skeletal muscle, cardiac C-protein is shown to be transiently expressed between Days 3 and 15 during development. In contrast, the expression of white skeletal C-protein is gradual and progressive starting approximately from Day 15 on in development. In primary cell cultures of skeletal muscle, however, cardiac C-protein remained expressed throughout prolonged culture time, this in conjunction with white skeletal C-protein. Thus the down regulation of cardiac C-protein and the transition from cardiac C-protein to adult skeletal (white) C-protein which was observed during skeletal muscle development *in vivo*, does not seem to go to completion in the *in vitro* system.

INTRODUCTION

Myogenic cells derived from embryonic muscle readily undergo terminal differentiation in culture for they align, fuse, and produce muscle-specific proteins. Finally, they exhibit cross-striation and spontaneously contracting muscle fibers with a full array of myofibrillar proteins (Konigsberg, 1963; Yaffe, 1968; Turner *et al.*, 1974, 1976; Emerson and Beckner, 1975; Paterson and Bishop, 1977; Merlie *et al.*, 1977; Strohman *et al.*, 1977; Devlin and Emerson, 1978; Perriard *et al.*, 1978).

Many studies have shown that a number of myofibrillar proteins exist in isoforms and that during muscle development a differential expression of these isoforms can take place. Such isoform transitions during myogenesis have been studied in detail, e.g., for creatine kinase, myosin heavy chains, myosin light chains, actin, troponin, and tropomyosin both *in vivo* and *in vitro* (Eppenberger *et al.*, 1964, 1967; Masaki and Yoshizaki, 1974; Turner *et al.*, 1974, 1976; Whalen *et al.*, 1976; Paterson and Eldridge, 1977; Dhoot and Perry, 1980; Matsuda *et al.*, 1981; Crow *et al.*, 1983; Toyota and Shimada, 1983). Even though in some cases there is some controversy on the number of existing isozymes of a given protein and on the exact order in which they are expressed during development, it is generally accepted that embryonic isoforms are first expressed in cultured cells derived from embryonic muscle tissue and that in culture often no or only incomplete transitions to the adult isoform patterns occur (Turner *et al.*, 1976; Matsuda *et al.*, 1981; Bandman *et al.*, 1982).

C-Protein, a myosin associated protein, is located at discrete positions along the thick filament (Offer *et al.*, 1973; Pepe and Drucker, 1975; Craig and Offer, 1976). Recently, it has been shown that distinct C-protein isoforms exist in white, red, and cardiac muscle (Jeacocke and England, 1980; Callaway and Bechtel, 1981; Reinach *et al.*, 1982; Starr and Offer, 1982; Yamamoto and Moos, 1983). The evidence for C-protein isoforms is based on analogies, like similarity in M_r, purification behavior, protein characteristics, and localization. No function has been attributed to C-protein as of yet. Cardiac C-protein was initially identified as a protein being phosphorylated *in vivo* by cAMP-dependent protein kinase (Jeacocke and England, 1980; Hartzell and Titus, 1982).

By using two monoclonal antibodies specific for fast- or slow-type skeletal muscle C-protein Obinata *et al.* (1984) studied the expression of these isoforms during chicken muscle development in the embryo. Both monoclonal antibodies reacted with pectoralis major (fast muscle) from late embryonic and young posthatched chickens. Slow-type C-protein decreased in amount progressively with age and was absent 2 weeks after hatching. Contrarily, in anterior latissimus dorsi (ALD; slow) only slow-type C-protein was detected throughout development starting from Day 17 *in ovo*.

In this communication we show by immunoblotting and immunofluorescence methods with polyclonal antibodies specific for cardiac or fast skeletal muscle C-

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protein that in the embryo the cardiac C-protein isoform is expressed initially both in embryonic skeletal and in heart muscle of avians. However, during later embryonic stages of skeletal muscle development cardiac C-protein is down regulated and is gradually replaced by C-protein isoforms that are specific for skeletal muscle, but cardiac C-protein remains the only C-protein isoform in heart muscle. The transition from cardiac C-protein to adult skeletal muscle C-protein which is observed during differentiation of embryonic skeletal muscle does not go to completion in cultured myogenic cells.

MATERIALS AND METHODS

C-Protein from chicken hearts (400 g) was prepared according to the method described for skeletal C-protein (Offer *et al.*, 1973). The procedure was slightly modified in that ammonium sulfate was added to 1.75 *M* instead of 1.66 *M* and that the solution for passing the sample over the DEAE-Sephadex A-50 column was adjusted to pH 7.9 and contained in addition 0.1 m*M* phenyl-methylsulfonyl-fluoride.

Antibodies were raised in rabbits by intracutaneous injection of 200 μg of cardiac C-protein (Fig. 1a), emulsified with complete Freunds adjuvant, twice at an interval of 2 weeks. Antiserum or affinity-purified IgG were used for the experiments. Characterization of antibodies against C-protein from chicken pectoralis muscle (anti-fast (white) skeletal muscle C-protein) will be described elsewhere (Bähler *et al.*, *J. Mol. Biol.*, in press). Ouchterlony double immunodiffusion tests were performed in 1.5% agar Noble dissolved in phosphate-buffered saline (PBS) supplemented with 0.4 *M* NaCl. Extracts were prepared by incubation for 30 min of well-washed myofibrils (pectoralis major, ALD, heart) with Guba-Straub solution (0.3 *M* KCl, 0.1 *M* KH_2PO_4, 0.05 *M* $KHPO_4$). Myofibrils were prepared as described earlier (Wallimann *et al.*, 1977). Electrophoresis and immunoblotting were performed according to Laemmli (1970) and Towbin *et al.* (1979), respectively.

Indirect immunofluorescence procedure: Chicken embryos were staged according to Hamburger and Hamilton (1951) and fixed in PBS containing 3% paraformaldehyde for about 1 hr. In embryos older than 7 days the breast region was excised and fixed separately. The fixed specimens were dehydrated first by ethanol followed by a xylol step and embedded in paraffin. Sections were cut 5–7 μm thick. Paraffin was removed by going through the dehydration procedure in reversed order. The sections were processed for indirect immunofluorescence after extensive rinsing in PBS.

Cells from breast muscle of 11-day-old embryos and from heart muscle of 8-day-old embryos were obtained and grown in culture (Turner *et al.*, 1974) and the immunofluorescence was performed as described (Wallimann *et al.*, 1977; Eppenberger *et al.*, 1981). For gel electrophoresis myogenic cells cultured for 4 days were scraped off the plate with a rubber policeman, directly put into gel sample buffer (Laemmli, 1970), and boiled for 5 min.

Tissue extracts were prepared by putting small tissue pieces in a solution containing 0.6 *M* NaCl, 10 m*M* $NaPO_4$, 1 m*M* dithiothreitol, 1 m*M* $MgCl_2$, 0.1 m*M* NaN_3 at pH 7.0. The suspension was thoroughly homogenized in a Polytron mixer (Kinematica, GmbH, Kriens, Switzerland), sonicated for 1 min, put into an equal volume of double-concentrated gel sample buffer (Laemmli, 1970), and boiled for 5 min.

RESULTS

The purity of the cardiac C-protein preparation is shown by $NaDodSO_4$-PAGE in Fig. 1a. Since C-protein is known to be highly antigenic (Offer, 1976) the immunization schedule was adjusted such that only twice

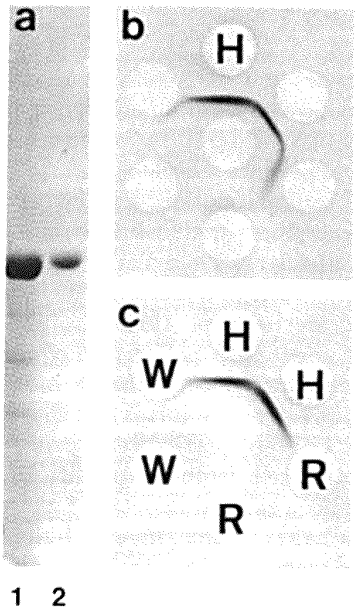

FIG. 1. Characterization of cardiac C-protein. (a) $NaDodSO_4$-polyacrylamide gel electrophoresis of purified cardiac C-protein. A 5% (w/v) polyacrylamide gel loaded with 35 μg (1) and 5 μg (2) of purified cardiac C-protein is shown after staining with Coomassie blue. (b and c) Double immunodiffusion of Guba-Straub extracts (outer wells) of myofibrils from heart (H); pectoralis major (W); and anterior latissimus dorsi (R) muscles, against cardiac C-protein antibodies (center wells). Specific rabbit anti-chicken cardiac C-protein antibodies (0.75 mg/ml) were placed in the central wells of plate b and c. In (b) the peripheral wells contained Guba-Straub extract of heart myofibrils (at 1 mg/ml in H), which was clockwise serially diluted. In (c) the peripheral wells contained Guba-Straub extracts of heart myofibrils at 0.75 mg/ml (H); pectoralis major myofibrils at 0.75 mg/ml (W); and anterior latissimus dorsi myofibrils at 0.75 mg/ml (R); H, W, and R standing for heart, white, and red muscle, respectively.

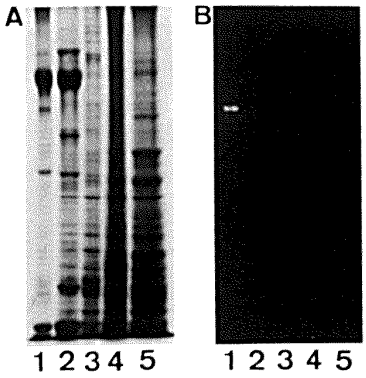

FIG. 2. Tissue specificity of heart C-protein. Washed and solubilized chicken heart myofibrils (1), and tissue homogenates of chicken gizzard (2), brain (3), kidney (4), and liver (5) were run on two identical 6% (w/v) polyacrylamide-$NaDodSO_4$ gels. The first gel was stained for protein with Coomassie blue (A). The second gel was transferred to nitrocellulose paper and incubated with rabbit anti-chicken cardiac C-protein antiserum (diluted by 1:50) followed by FITC-conjugated goat anti-rabbit IgG (diluted by 1:100) as a second antibody.

small quantities of protein (150–200 μg) were injected into the rabbits. Thus reducing the chance of eliciting antibodies to any potential minor contaminants in the cardiac C-protein preparation. The specificity of the obtained sera for cardiac C-protein is shown in Figs. 1b, 2, and 3. In Ouchterlony double immunodiffusion tests the anti-cardiac C-protein antiserum gave only one single, strong precipitin line with a Guba-Straub extract of chicken heart myofibrils (Fig. 1b) and none with similar extracts from chicken pectoralis major (fast, white) or anterior latissimus dorsi (ALD, slow, red) myofibrils (Fig. 1c).

Chicken heart myofibrils stained, after blotting from $NaDodSO_4$ gels onto nitrocellulose, with anti-cardiac C-protein antiserum followed by FITC-conjugated second antibody one single band of apparent M_r of 165,000 that was strongly labeled (Fig. 2B, lane 1). No cross-reactivity of the anti-cardiac C-protein antibody was found by this method with homogenates from adult chicken gizzard, brain, liver, or kidney (Fig. 2). In addition, no cross-reactivity of anti-cardiac C-protein antibodies with C-protein from slow or fast skeletal muscle was observed by immunoblotting either (Fig. 3).

Compared with C-protein from fast skeletal muscle which is shown here by immunoblotting with anti-pectoralis C-protein antibodies to have an apparent M_r of 140,000 (Offer *et al.*, 1973) (Fig. 3C), cardiac C-protein exhibited a somewhat higher M_r of approximately 165,000 which is in accordance with the current literature (Hartzell and Titus, 1982; Yamamoto and Moos, 1983) (Fig. 3B, and lane 4). The lack of cross-reactivity of anti-cardiac C-protein antibodies with C-protein from skeletal muscle was also confirmed by indirect immunofluorescence staining of isolated myofibrils (Fig. 4). Anti-cardiac C-protein antibody strongly stained the middle third of each A-band halves of adult cardiac myofibrils (Fig. 4b) and gave a cross-striated pattern in cardiac cells in culture (Fig. 4f), but no staining whatsoever of skeletal muscle myofibrils was observed with the same antibody (Fig. 4d). These results strongly suggest that the antibody used is specific for cardiac C-protein and that, in the adult chicken, cardiac C-protein represents a protein found exclusively in heart, but not in skeletal muscle, gizzard, kidney, liver, or brain (Fig. 2). Surprisingly, however, in myogenic cell cultures, cardiac C-protein was detected both by immunoblotting (Fig. 3) as well as by immunofluorescence staining (Fig. 5). Anti-cardiac C-protein antibody stained on a blot of an extract of cultured myogenic cells a protein with identical electrophoretic mobility (M_r 165,000) as cardiac C-protein (Fig. 3B, faint band in lane 3). If a parallel blot was incubated with anti-pectoralis C-protein antibody an extremely faint band (Fig. 3C, lane 3) with identical electrophoretic mobility as fast skeletal muscle C-protein (Fig. 3C, lane 1, M_r = 140,000) was detected, but only by employing significantly higher concentrations of anti-pectoralis C-protein antibodies and longer incubation times as compared with the anti-cardiac C-protein blots (Fig. 3B). No additional band in the M_r-range between 130 and 170K, that is, no presumable embryonic isoform of C-protein was detected with either anti-cardiac or anti-fast skeletal C-protein antibodies (Fig. 3). The expression of cardiac C-protein in myogenic cells which

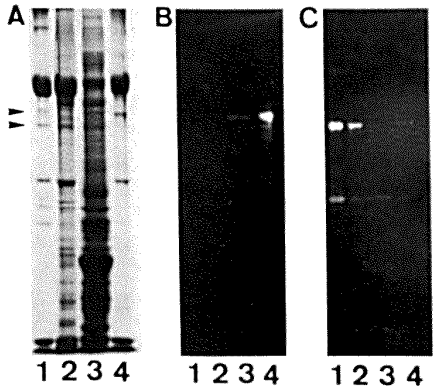

FIG. 3. Immunoblot analysis of C-protein isoforms. Myofibrils from adult pectoralis major (white) (1); anterior latissimus dorsi (red) (2); heart (4); and solubilized myogenic cells (3), derived from embryonic breast muscle cultured for 4 days were run on three identical 6% polyacrylamide-$NaDodSO_4$ gels. The first gel (A) was stained for protein with Coomassie blue, the second gel (B), after transfer to nitrocellulose, was incubated with rabbit anti-cardiac C-protein IgG followed by FITC-conjugated goat anti-rabbit IgG. The third gel (C), after transfer to nitrocellulose, was stained with rabbit anti-white skeletal muscle C-protein antiserum. Arrowheads indicate the position of cardiac (M_r = 165,000) and white skeletal (M_r = 140,000) C-protein.

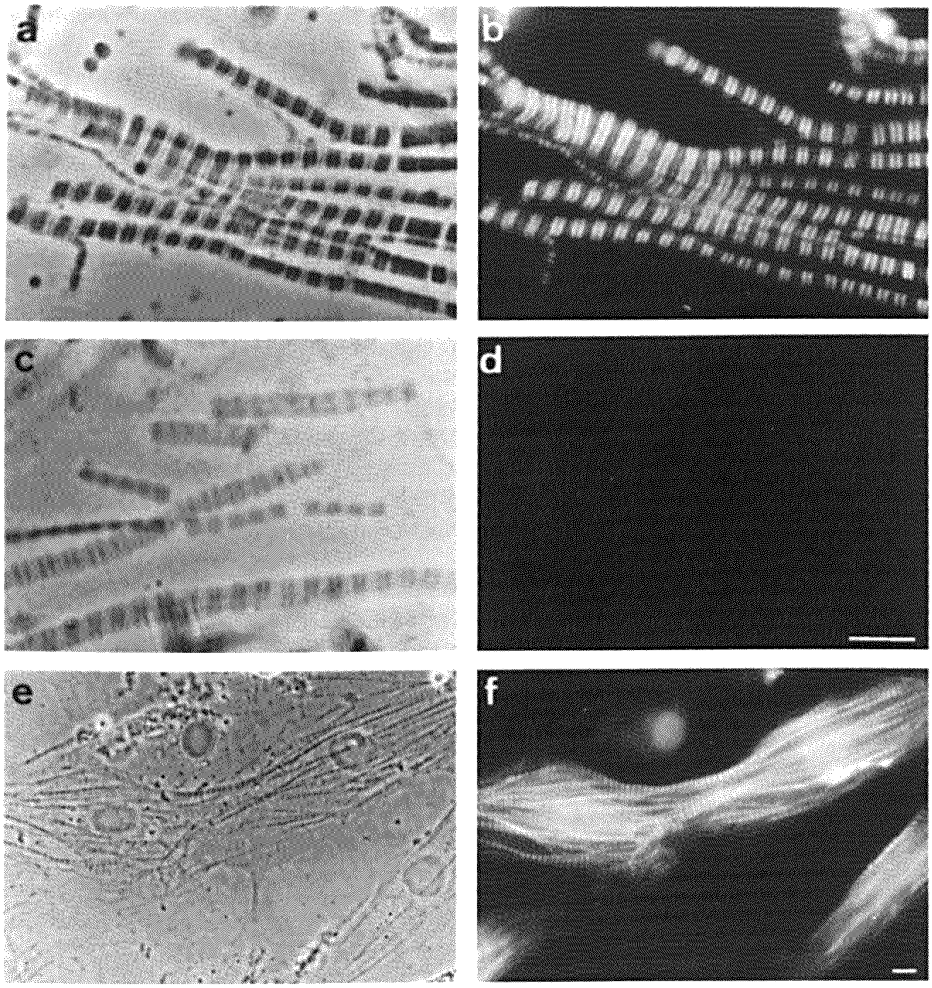

FIG. 4. Immunofluorescence staining of isolated myofibrils and cultured embryonic heart muscle cells. Indirect immunofluorescence staining of isolated heart (a, b) and pectoralis major (c, d) myofibrils with affinity-purified anti-cardiac C-protein IgG (8 μg/ml). Heart cells derived from embryonic heart muscle cultured for 3 days shown after incubation with anti-cardiac C-protein antiserum followed by FITC-conjugated second antibodies (e, f). The photographs are paired phase contrast (left) and fluorescence images (right). Bar, 8 μm.

were derived from embryonic chicken skeletal muscle was further investigated by immunofluorescence staining (Fig. 5).

Little fluorescence staining was seen with anti-cardiac C-protein antibody in myogenic cells cultured for 1 day (Fig. 5b). Only occasionally, a mononucleated, presumably postmitotic myoblast showed already a cross-striated fiber-like pattern (Fig. 5b). However, skeletal muscle myotubes after 2 days in culture showed a regular, cross-striated fluorescent labeling pattern when stained by anti-cardiac C-protein antibody (Fig. 5d) whereas in myotubes that are fully packed with bundles of myofibrils after 11 days in culture clear labeling of each A-band-half was discerned (Fig. 5f). This labeling corresponded to that one found on isolated adult cardiac myofibrils (Fig. 4b) or cardiac cells in culture (Fig. 4f) after staining with anti-cardiac C-protein antibody. Indirect immunofluorescence labeling of skeletal myotubes by anti-skeletal C-protein antibody revealed a similar staining pattern (Fig. 5h).

Detection of cardiac C-protein in primary skeletal muscle cell cultures paralleled the formation of myofibrils and cardiac C-protein did not seem to be down regulated in these cultures even after prolonged time in culture. Although fast skeletal muscle C-protein was being accumulated, no complete transition from cardiac C-protein to skeletal C-protein as seen in embryonic muscle (Fig. 6) seemed to occur in these cells cultured *in vitro* under standard conditions.

By contrast, during skeletal muscle development *in vivo* the cardiac form of C-protein, although accumulated prior to the other C-protein isoforms, is only transiently

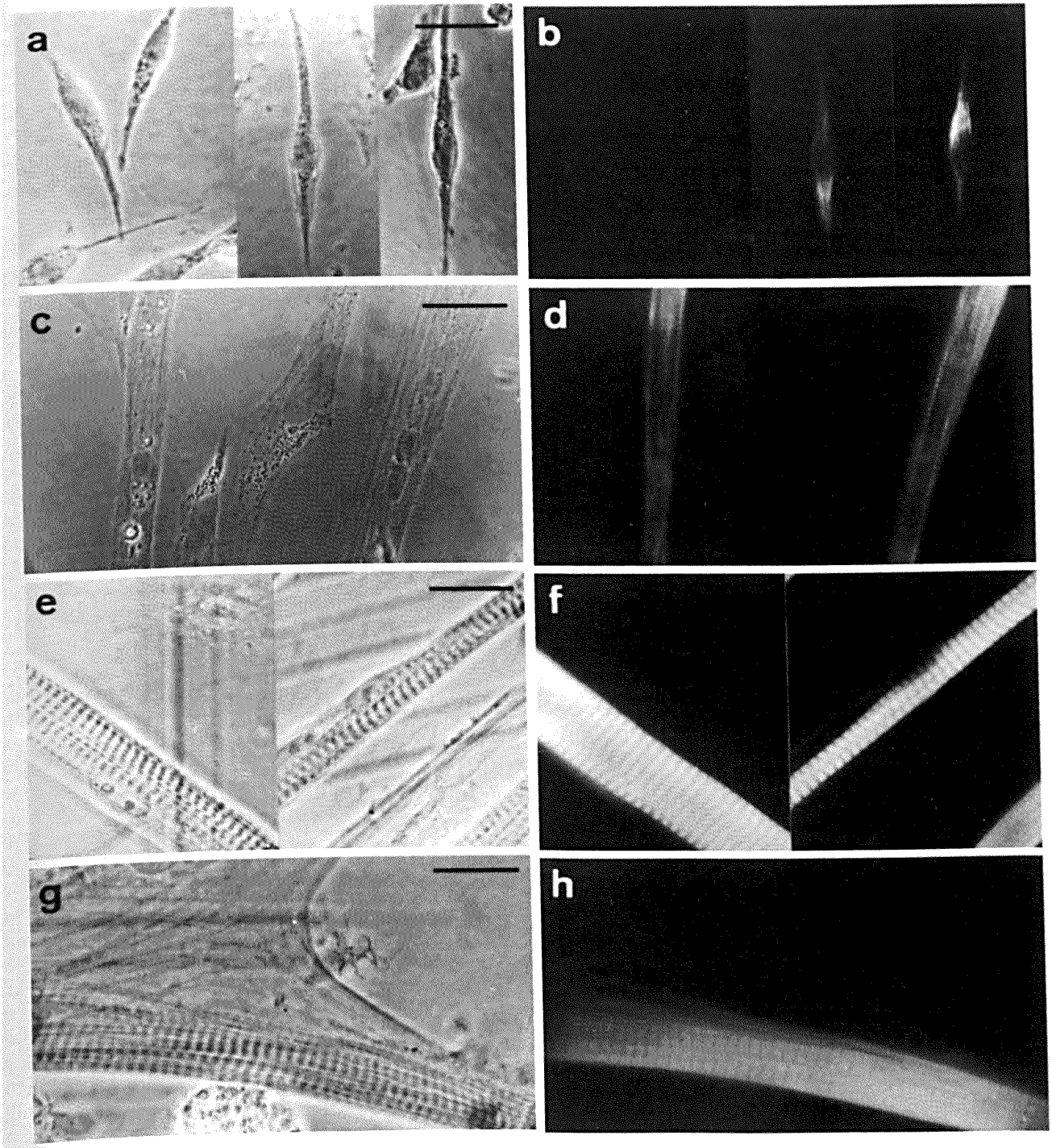

FIG. 5. Appearance of cardiac and skeletal C-protein in developing myogenic cells *in vitro*. Indirect immunofluorescence staining of cultured skeletal muscle cells with anti-cardiac C-protein (a–f) or anti-fast C-protein (g, h) antibodies. Myogenic cells derived from embryonic chicken breast muscle cultured for 1 day (a, b); 2 days (c, d); and 11 days (e–h). Phase-contrast (left) and fluorescence images (right) are paired. Bar, 20 μm.

expressed between Days 3 and 15 (Fig. 6) whereas skeletal C-protein is expressed at much later stages from Days 15 to adult (Fig. 6). On the other hand, cardiac C-protein is the only C-protein isoform found in developing heart cell cultures as well as in embryonic and in adult chicken heart tissue. Thus, in developing heart,

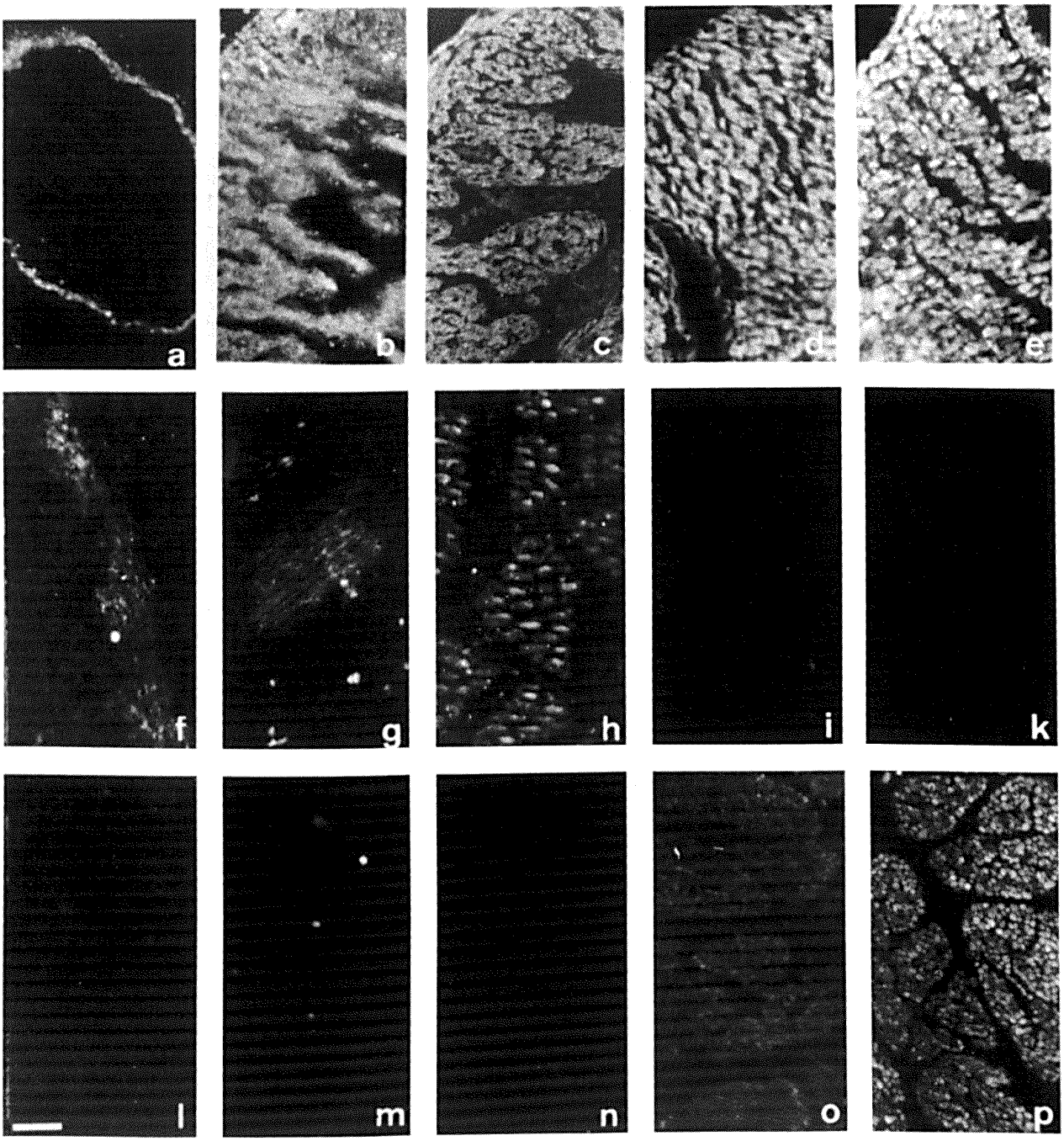

FIG. 6. Appearance of C-protein isoforms during the development of the chick embryo. Indirect immunofluorescence staining of embryonic heart (a–e); embryonic myotomes (f, g, l, m); or breast muscle (h, i, k, n, o, p) with antibodies against cardiac C-protein (a–k;) and against fast skeletal muscle C-protein (l–p). Paraffin sections of hearts from 3-day-old (Hamburger–Hamilton; stage 17) in (a); 7-day-old (b); 12-day-old (c); 15-day-old (d); and 19-day-old (e) chicken embryos after incubation with rabbit anti-cardiac C-protein antibodies followed by FITC-conjugated second antibody. Serial sections through myotomes of 3-day-old (H, H-stage 19) (f, l); and 7-day-old chicken embryos (g, m); through pectoralis major muscle from 12-day-old (h, n); 15-day-old (i, o); and 19-day-old chicken embryos (k, p) after incubation with anti-cardiac C-protein (f–k) or anti-fast C-protein (l–p) antibodies followed by FITC-conjugated second antibody. Sections were photographed on Ilford HP-5 film under epifluorescence illumination. Bar, 100 μm.

the cardiac C-protein isoform persists from early embryonic stages (before Day 3 in ovo) throughout adulthood.

DISCUSSION

Expression of Cardiac Isoproteins in Developing Skeletal Muscle

The presence of cardiac C-protein in cultured cells from skeletal muscle as well as in early embryonic skeletal muscle was demonstrated by indirect immunofluorescence and immunoblotting using antibodies specific for cardiac C-protein. The antibody was prepared against C-protein from adult chicken hearts and stained the middle third of both A-band-halves of isolated heart, but not skeletal myofibrils from adult chickens. The antibody showed no cross-reactivity with other C-protein isoforms, e.g., C-proteins from adult fast or slow skeletal muscle. This is in accordance with the results of Yamamoto and Moos (1983) who found no immunological cross-reactivity of anti-fast skeletal muscle C-protein antibodies with cardiac C-protein. Also, a lack of immunological cross-reactivity between C-protein isoforms from fast and slow skeletal muscle has been shown earlier (Callaway and Bechtel, 1981; Reinach *et al.*, 1982; Yamamoto and Moos, 1983).

The C-protein isoforms are also known to differ in their apparent M_r (Yamamoto and Moos, 1983). According to our results, in embryonic skeletal muscle and myogenic cell cultures, a protein of identical M_r to cardiac C-protein was found to react with the antibody against cardiac C-protein. Therefore, we feel confident that the protein detected during early skeletal muscle development is cardiac C-protein and not an embryonic C-protein isoform. This finding fits well into recent data obtained with other myofibrillar proteins, that is, cardiac isoforms of myosin heavy chain (Sweeney *et al.*, 1984), myosin light chains (Whalen *et al.*, 1982), troponin T and C (Toyota and Shimada, 1981, 1983), and actin (Paterson and Eldridge, 1984) all of which have been demonstrated in cultured skeletal muscle cells as well as in somitic myotomes. Thus, the expression of cardiac C-protein in early skeletal muscle adds another example to these findings. However, it is the first one described for a thick filament associated protein. The results are consistent with the recent proposal made by Sweeney *et al.* (1984) who suggests that the primordial or epithelial stage of striated muscle persists in the myocardium and is represented by the presence of cardiac myosin, while it is only transiently present in the early stages of embryonic skeletal muscle development. We conclude that C-protein expression occurs much earlier in development than was thought before by Obinata *et al.* (1984). For this reason, *in vitro* studies with isolated proteins aiming at the elucidation of the role of C-protein in thick filament assembly may be more promising if conducted with C-protein from adult heart than with C-protein from adult skeletal muscle, for cardiac C-protein seems to be expressed concomittantly with myofibrillogenesis.

Incomplete Transition of C-Protein Isoforms in Vitro

Cardiac C-protein is detected in embryonic heart muscle as well as in cultured heart muscle cells at very early stages, e.g., at 2–3 days in ovo, and at all times in culture. It remains the only C-protein isoform found in heart from the beginning throughout adulthood. However, in contrast to the embryonic development of skeletal muscle where cardiac C-protein was found to be expressed only transiently, cardiac C-protein seems to persist in skeletal muscle cell cultures *in vitro* even after prolonged periods of culturing. While during skeletal muscle development in the embryo cardiac C-protein, although appearing at early stages (from Day 3 on), is gradually down regulated and replaced by skeletal muscle C-proteins at around Days 15–17 of embryonic skeletal muscle development, cardiac C-protein remains expressed in myogenic cells in tissue cultures. Thus, in contrast to the embryonic situation, where during skeletal muscle differentiation a transition of C-protein isoforms from cardiac to fast and/or slow skeletal C-protein occurs, no such transition or only a very incomplete one is observed in the homologous tissue culture system. Since a complete C-protein isoenzyme transition occurs during embryonic muscle development, but not in culture and since in differentiated skeletal muscle the C-protein isoforms are expressed in a fiber type-specific way (Callaway and Bechtel, 1981; Reinach *et al.*, 1982), specific antibodies against C-protein isoforms may turn out to be valuable tools for studying terminal differentiation of myogenic cells under a variety of culture conditions, e.g., direct electric stimulation, coculturing with nerve cells, or addition of humoral factors. Very recently it has been shown that muscle denervation markedly affects the expression of C-protein isoforms (Obinata *et al.*, 1984b). Since denervation does not seem to alter myosin isoform expression in parallel with C-protein it was suggested by these authors that these two proteins are regulated independently by the nervous system. Thus, using the C-protein transition as an experimental system it will be very interesting to find out more about the factors involved in terminal muscle differentiation and muscle fiber type specification.

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