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Induction and regulation of connexin26 by glucagon in primary cultures of adult rat hepatocytes

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SUMMARY

In the adult rat hepatocyte, the gap junction proteins consist of a major component, connexin32 (Cx32) and a minor component, connexin26 (Cx26). Although we recently reported our success in inducing and maintaining Cx32 in adult rat hepatocytes cultured in serum-free L-15 medium supplemented with epidermal growth factor and 2% dimethyl sulfoxide, it was very difficult to induce Cx26 in the primary hepatocytes. In the present study, we found that the addition of 10^{-7} M glucagon into the culture medium could dramatically induce Cx26 mRNA and protein. Although the expression of Cx32 mRNA was also influenced by glucagon, the increase of the expression was small. Immunocytochemically, Cx26-positive spots were observed between most adjacent cells and were co-localized with the Cx32-positive spots. We also examined whether 0.5 mM dibutyl cyclic AMP could induce expression of Cx26 in the cells. The effect of dexamethasone on the expression of Cx26 mRNA compared to that of Cx32 mRNA was examined. For the induction and maintenance of Cx26 mRNA, more than 10^{-7} M dexamethasone was necessary in this culture. These results suggest that expression of Cx26 in hepatocytes may be regulated by the concentrations of glucagon and glucocorticoid hormones.

Key words: connexin26, rat hepatocyte, glucagon, dexamethasone, primary culture

INTRODUCTION

Gap junctions are intercellular membrane channels that link neighboring cells and mediate reciprocal exchanges of small molecules of less than 1,000 daltons and ions, including second messengers such as cAMP, inositol triphosphate and Ca^{2+}, between adjacent cells in contact (Sáez et al., 1986, 1989a; Spray et al., 1986, 1988). Gap junctional intercellular communication (GJIC) is thought to play a crucial role in the maintenance of homeostasis, morphogenesis, cell differentiation and growth control in multicellular organisms (Loewenstein, 1979; Bennett et al., 1991; Lang et al., 1991; Mesnil and Yamasaki, 1993). Gap junctions are composed of proteins called 'connexins' (Haefliger et al., 1992). In rat hepatocytes, both Cx26 and 32 (Nicholson et al., 1987) are expressed, and the distribution of these Cx proteins is reported to be different within the liver lobule: Cx26 preferentially localizes in the periportal zone of the lobules, whereas Cx32 appears in most hepatocytes throughout the lobules (Sakamoto et al., 1992; Traub et al., 1989). In addition, it is known that the relative ratios of Cx26 to Cx32 of protein and mRNA in rat livers are 1:10 and 1:50, respectively (Nicholson et al., 1987; Zhang and Nicholson, 1989). However, the specific functions and the regulation of the Cxs in hepatocytes are still unclear. The most critical reason why the mechanisms of Cxs expression are not sufficiently investigated in hepatocytes is that the induction and maintenance of gap junctions are very difficult and that the expression of mRNA and proteins of Cxs is very weak. Spray et al. (1987) showed that extracellular matrix components such as proteoglycans and glycosaminoglycans could induce the synthesis and expression of Cx32. Futhermore, dibutyl cyclic AMP (dib-cAMP) was shown to increase the stability of Cx32 mRNA and to delay the disappearance of gap junctions in cultured hepatocytes (Sáez et al., 1989b; Bennett et al., 1991). However, the induction and the regulation of Cx26 in primary rat hepatocytes has never been reported.

Recently, we succeeded in inducing and maintaining the gap junctional protein Cx32 in adult rat hepatocytes cultured in serum-free L-15 medium supplemented with epidermal growth factor (EGF) and dimethylsulfoxide (DMSO) (Kojima et al., 1995). In the present experiment we showed that by the addition of glucagon to the medium, Cx26 could also be induced and maintained for more than 2 weeks. The effect of dib-cAMP and dexamethasone on the expression of Cx26 compared to Cx32 was also examined.

MATERIALS AND METHODS

Isolation and culture of rat hepatocytes

Male Sprague-Dawley rats (Shizuoka Laboratory Animal Center, Hamamatsu, Japan) weighing about 300-400 g were used to isolate

hepatocytes by the two-step liver perfusion method of Seglen (1976) with some modification (Mitaka et al., 1991). Briefly, the liver was perfused in situ through the portal vein with 150 ml of Ca^{2+}, Mg^{2+}-free Hanks' balanced salt solution (HBSS) supplemented with 0.5 mM EGTA (Sigma Chemical Co., St Louis, MO), 0.5 mg/l insulin (Sigma) and antibiotics. After the initial brief perfusion, the liver was perfused with 200 ml HBSS containing 40 mg of collagenase (Yakulto Co., Tokyo, Japan) for 10 minutes. The isolated cells were purified by Percoll iso-density centrifugation (Kreamer et al., 1986). Viability of the cells by the trypan blue exclusion test was more than 90% in these experiments. The cells were suspended in L-15 medium with 0.2% bovine serum albumin (BSA; Seikagaku Kogyo Co., Tokyo, Japan), 20 mM HEPES (Dojindo, Kumamoto, Japan), 0.5 mg/l insulin (Sigma), 10^{-7} M dexamethasone (Sigma), 1 g/l galactose (Sigma), 30 mg/l proline (Sigma), and antibiotics. The isolated hepatocytes were plated on 35 mm and 60 mm culture dishes (Corning Glass Works, Corning, NY), which were coated with rat tail collagen (500 μg dried tendon/ml of 0.1% acetic acid) (Michalopoulos and Pitot, 1975), and placed on a 100% air incubator at 37°C. Two to three hours after plating, the medium was changed to L-15 medium supplemented with 0.2% BSA, 20 mM HEPES, 0.5 mg/l insulin, 10^{-7} M dexamethasone, 1 g/l galactose, 30 mg/l proline, 20 mM $NaHCO_3$, 5 mg/l transferrin (Wako Pure Chemical Inc., Osaka, Japan), 0.2 mg/l $CuSO_4{\cdot}5H_2O$, 0.5 mg/l $FeSO_4{\cdot}4H_2O$, 0.75 mg/l $ZnSO_4{\cdot}7H_2O$, 0.05 mg/l $MnSO_4$, 5 μg/l Na_2SeO_3, 10 ng/ml EGF (Becton Dikinson Labware, MA), and antibiotics. The cells were then placed in a humidified, 5% CO_2:95% air incubator at 37°C. The medium was replaced with fresh medium every other day, and 2% DMSO (Aldrich Chemical Co. Inc., Milwaukee, WI) was added to the medium after 96 hours of culture. After some dishes were cultured in 2% DMSO medium supplemented with glucagon (glucagon novo, Yamanouchi, Tokyo, Japan) or dib-cAMP (Sigma) from 96 hours after plating, the cells were fixed or harvested.

Northern blot analysis and densitometry

Total RNA was extracted from the cells, using the single-step thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987) as modified by Xie and Rothblum (1991). For electrophoresis, 10 μg of total RNAs was loaded on a 1% agarose gel containing 0.5 mg/l ethidium bromide. Gels were capillary-blotted in 20× standard saline citrate (SSC) onto a nylon membrane (Hybond-N; Amersham Corp., Buckinghamshire, UK) and fixed by ultraviolet light. For the detection of Cx26 mRNA, Cx32 mRNA and Cx43 mRNA, digoxigenin (DIG)-labeled RNA probes were prepared from rat Cx26 cDNAs (Zhang and Nicholson, 1989), rat Cx32 cDNAs (Paul, 1986), and rat Cx43 cDNAs (Beyer et al., 1987) using an RNA labeling kit (Boehringer Mannheim, Mannheim, Germany). Hybridization, washing and chemiluminescent detection were carried out following the DIG luminescent protocol (Höltke et al., 1992). Scanning-densitometry was performed using a Macintosh Quadra 800 computer (Apple Computer, Cupertino, CA) and an EPSON GT-6000 scanner (Seiko Epson, Suwa, Japan). The signals were quantified by the NIH Image 1.52 Densimetric Analysis Program (Wayne Rasband, NIH, Bethesda, MD) (Masters et al., 1992). Expression of the transcripts was shown as a percentage of 0 hour (isolated hepatocytes) values in the same experiment, which was demonstrated as a histogram. Ethidium bromide staining of ribosome RNAs was shown to confirm the presence of equivalent loading of total RNAs. To examine the amounts of loading RNAs, we also used β-actin cDNA probe (Wako) in this experiment. The results were similar to that of the ethidium bromide staining. The results are representative of three separate experiments.

Western blot analysis

The dishes were washed with PBS twice and 1 ml of the buffer (1 mM $NaHCO_3$, 20 mM NaOH and 2 mg/l leupeptin (Sigma)) was added to 60 mm dishes. The cells were scraped and collected in Eppendorf tubes and then sonicated for 30 seconds. The sonicates were centrifuged at 12,000 ***g*** for 30 minutes. The pellets were washed with 20 mM NaOH and then 1 mM $NaHCO_3$ was added for neutralization, using centrifugation conditions. NaOH-insoluble pellets were resuspended in the buffer (250 mM Tris-HCl (pH 6.8), 10% SDS and 50% glycerol). The protein concentration of the samples was determined using a protein assay kit (Pierce Chemical Co., Rockford, IL). 20 μg of protein of each sample per lane was applied and separated by electrophoresis in 12.5% SDS-polyacrylamide gel (Daiichi Pure Chemicals Co., Tokyo, Japan). After electrophoretic transfer to a nitrocellulose membrane (Bio-Rad, Richmond, CA) using semi-dry blotting for 6 hours (0.65 mA/cm^2), the membrane was saturated overnight at 4°C with a blocking buffer (25 mM Tris-HCl, pH 8.0, 125 mM NaCl, 0.1% Tween-20 and 4% skim milk) and incubated with a rabbit anti-rat Cx26 polyclonal antibody (Kuraoka et al., 1993) at room temperature (RT) for 2 hours. The membrane was incubated with a horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Vector Laboratories, Burlingame, CA) and the detection was carried out using an enhanced chemiluminescence (ECL) Western blotting system (Amersham Corp., Buckinghamshire, UK).

Immunofluorescence microscopy

The cells grown on coated glass coverslips (BIOCOAT, Becton Dickinson Labware) were fixed with acetone for 30 minutes at −20°C. After rinsing with phosphate-buffered saline (PBS), the coverslips were incubated at RT for 1 hour with a rabbit anti-rat Cx26 polyclonal antibody or a mouse monoclonal anti-rat Cx32 antibody (Goodenough et al., 1988). The cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse or anti-rabbit IgG (DAKO, Copenhagen, Denmark) at RT for 1 hour and were examined with a Nikon Fx epifluorescence photomicroscope (Nikon, Tokyo, Japan). Some coverslips were used for double staining for Cx26 and Cx32. FITC-conjugated anti-rabbit IgG (DAKO) for Cx26 staining and horse Texas Red-conjugated anti-mouse IgG (Vector Laboratories) for Cx32 staining were used. The samples were photographed in a MRC-600 confocal laser microscope (Bio-Rad).

Measurement of GJIC

For measuring GJIC, we used the scrape loading/dye transfer method (El-Fouly et al., 1987) with some modification. The hepatocytes on 35 mm dishes were rinsed several times with PBS. Two or three lines were made around the center of the dish using a surgical blade and 2 ml of 0.05% Lucifer Yellow CH (LY; Sigma) in PBS was added. LY is a small molecule (457 Da) which can freely move through gap junctions from loaded cells to neighboring ones. Three minutes after the dye treatment, the cells were rinsed several times with PBS to remove excess dye. We used rhodamine dextran, which is known not to move through gap junctions, as a control. We immediately observed the intensity of LY transfer with an Olympus inversed microscope equipped with appropriate filters (Olympus, Tokyo, Japan) and photographed it. We carried out at least three separate experiments. The results shown here are reproducible.

RESULTS

Cx mRNA expression of primary rat hepatocytes

We recently showed that primary cultured hepatocytes could reexpress and maintain the gap junctional protein Cx32 in adult rat hepatocytes cultured in serum-free modified L-15 medium supplemented with 2% DMSO, and that the changes of expression of Cx32 are controlled at the mRNA level (Kojima et al., 1995). Fig. 1 shows the changes in transcripts of mRNAs of connexins in hepatocytes treated with (+) or without (−) 2%

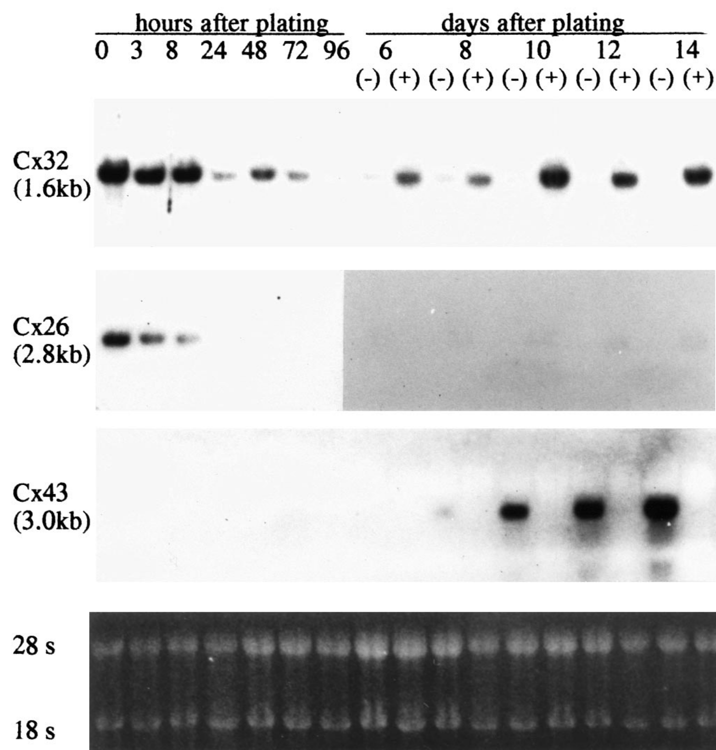

Fig. 1. Northern blot analysis for Cx32, 26 and 43 of primary rat hepatocytes cultured in modified L-15 medium with (+) or without (–) 2% DMSO. The left half shows samples from 0 hours (isolated hepatocytes) to 96 hours without 2% DMSO. The right half shows samples from day 6 to day 14 after addition of 2% DMSO from 96 hours. Total RNA (10 μg/lane) was fractionated by electrophoresis in a 1% agarose-formaldehyde gel and hybridized with digoxigenin-labeled Cx32, 26 and 43 cRNA probes. The lower panel shows ethidium bromide staining of ribosome RNAs before transfer to membranes.

DMSO from 96 hours after plating. As previously described, the expression of Cx32 mRNA gradually decreased until 96 hours, and after 2% DMSO was added to the medium at 96 hours, the expression of Cx32 mRNA increased and was maintained at the same high level as that of the hepatocytes at 3 hours after plating. On the other hand, without DMSO the expression of Cx26 mRNA rapidly decreased and was not observed after 24 hours. In the hepatocytes treated with 2% DMSO from 96 hours, the expression of Cx26 mRNA reappeared at day 6, but it was very low and unstable compared to that of Cx32. Futhermore, immunocytochemically, Cx26-positive spots were rarely observed in the hepatocytes (data not shown). Cx43 mRNA was observed after day 8 and the expression increased with time in culture when the cells were cultured without DMSO. This expression of Cx43 mRNA in primary cultures of hepatocytes has usually been attributed to contaminating non-parenchymal cells (Stutenkemper et al., 1992), and in fact Cx43-positive spots were immunocytochemically seen only in non-parenchymal cells (data not shown).

Effects of glucagon and dib-cAMP on the expression of Cx26 and Cx32 mRNA

As glucagon and dib-cAMP were reported to delay the disappearance of gap junctions in primary cultures of hepatocytes (Sáez et al., 1989b; Bennett et al., 1991), we examined whether they were effective in the induction of both Cx26 and Cx32 mRNAs. In this experiment, the cells cultured with the medium contained 2% DMSO, 10^{-7} M dexamethasone and 0.5 mg/l insulin were treated with either glucagon or dib-cAMP from 96 hours after plating. The effects of various concentrations of glucagon on the expression of Cx26 and 32 mRNAs are shown in Fig. 2. Cx26 mRNA markedly increased at doses of more than 10^{-9} M glucagon at day 6 and at doses of more than 10^{-8} M at day 10, but Cx32 mRNA only slightly increased at a dose of 10^{-7} M at day 10. As both Cx26 and Cx32 mRNA were most induced at a dose of 10^{-7} M glucagon, the changes of these mRNAs were examined during the culture. The amount of Cx26 mRNA in the cells treated with 10^{-7} M glucagon increased about 2-fold as much as that in the cells without glucagon, while the amount of Cx32 mRNA in the cells with glucagon slightly increased (Fig. 3).

To investigate whether dib-cAMP could substitute for glucagon, the hepatocytes were cultured in 2% DMSO medium supplemented with 0.5 mM dib-cAMP from 96 hours after plating. The cells treated with dib-cAMP expressed much higher levels of Cx26 mRNA than those without dib-cAMP at both days 6 and 10, while Cx32 mRNA slightly increased in the cells treated with dib-cAMP at day 10 (Fig. 4).

Effect of dexamethasone on the expression of Cx26 and Cx32 mRNA

Glucocorticoids such as dexamethasone are well known to regulate the expression of many liver genes in vitro. In this experiment we examined the effect of dexamethasone on the induction of both Cx26 and Cx32 mRNA. Various concentrations of dexamethasone combined with or without 10^{-7} M glucagon were added to the culture medium supplemented with 2% DMSO from 96 hours. At days 6 and 10, 10^{-5} M dexamethasone enhanced the expression of Cx26 mRNA in the hepatocytes treated with glucagon, while without glucagon we observed a slight increase of the expression at the concentration of 10^{-5} M (Fig. 5A). For the induction of Cx26 mRNA, we found that more than 10^{-7} M dexamethasone was necessary in the culture medium. The expression of Cx32 mRNA was

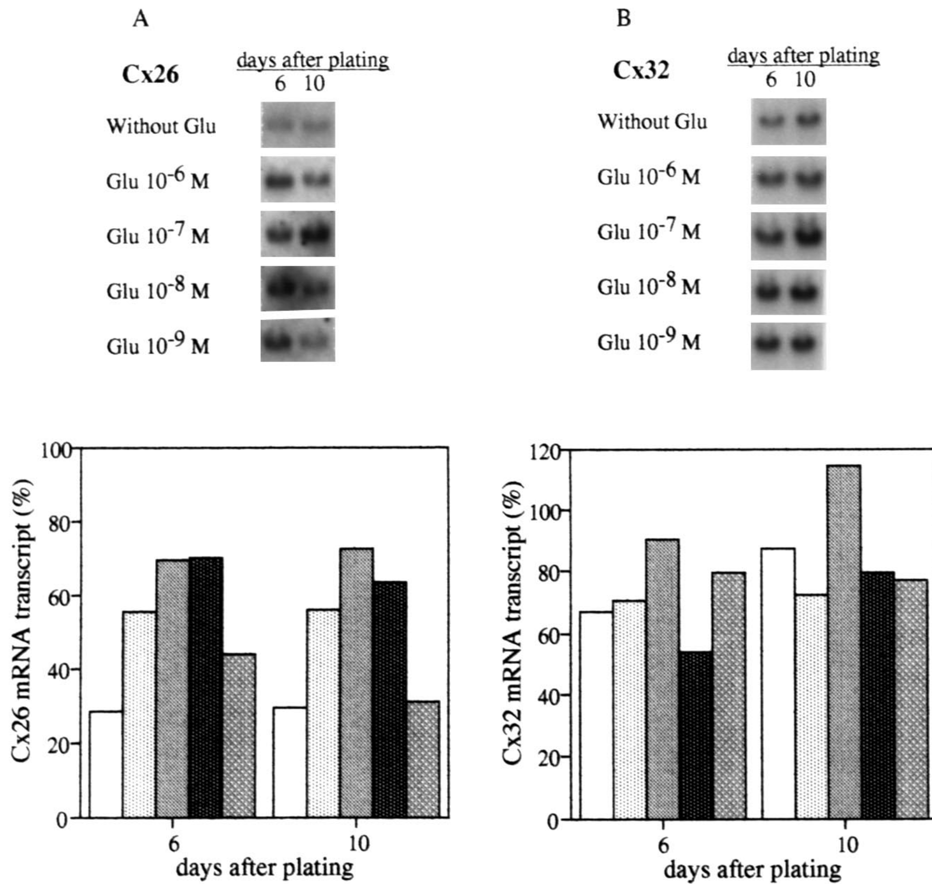

Fig. 2. Effects of the concentration of glucagon on Cx26 (A) and Cx32 mRNA (B) transcripts. The medium was changed to 2% DMSO medium with or without glucagon from 96 hours. Scanning-densitometric analysis of the mRNA level was performed. Details are described in Materials and Methods. The results are shown in the histograms (A,B); □: without glucagon, ▒: with 10^{-6} M glucagon, ▒: with 10^{-7} M glucagon, ▩: with 10^{-8} M glucagon, ▩: with 10^{-9} M glucagon. Expression of the transcripts is shown as the percentage of 0 hour values.

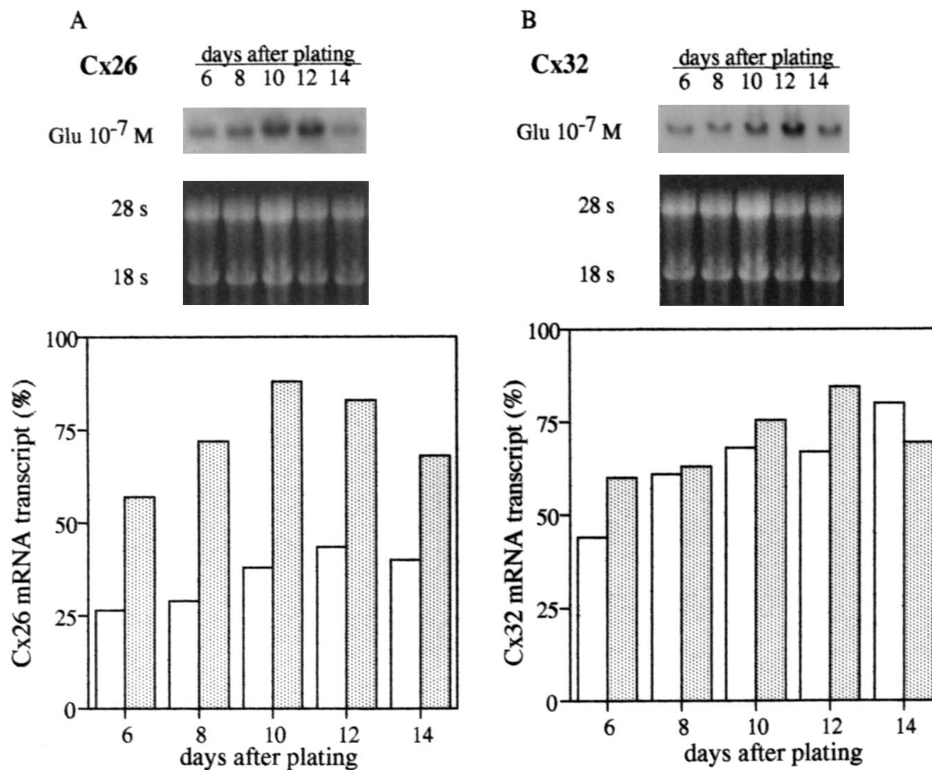

Fig. 3. Effects of 10^{-7} M glucagon on Cx26 (A) and Cx32 mRNA (B) transcripts. The medium was changed to 2% DMSO medium with or without 10^{-7} M glucagon from 96 hours. Glu: glucagon. The results are shown in the histograms (A,B); □: without glucagon in Fig. 1, ▒: with glucagon. Expression of the transcripts is shown as the percentage of 0 hour values.

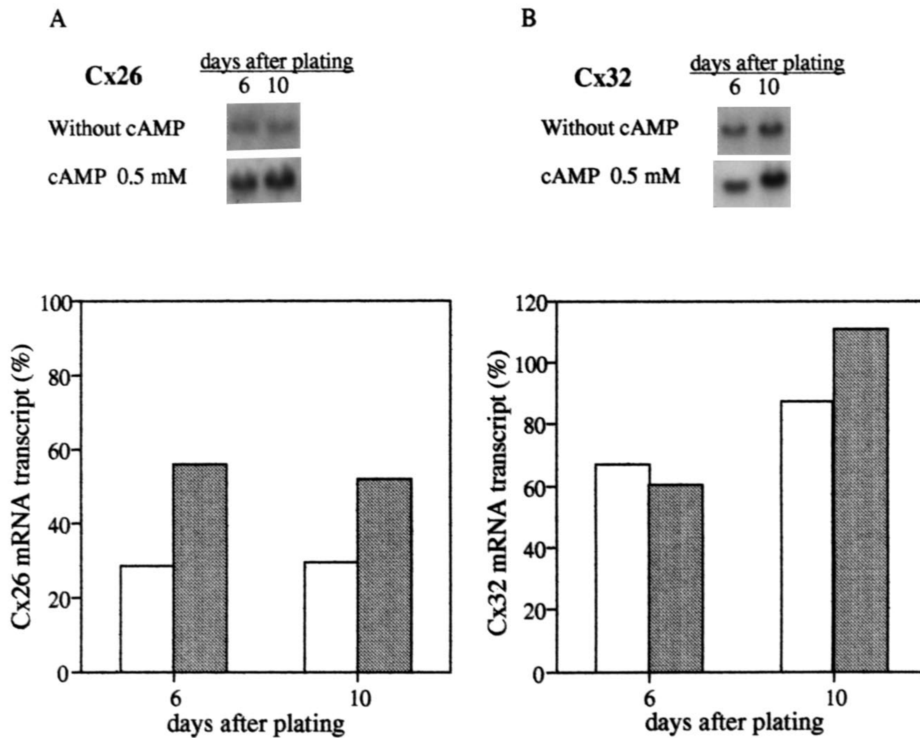

Fig. 4. Effects of 0.5 mM dib-cAMP on Cx26 (A) and Cx32 mRNA (B) transcripts. The medium was changed to 2% DMSO medium with or without 0.5 mM dib-cAMP from 96 hours. The results are shown in the histograms (A,B); □: without cAMP, ▥: with cAMP. Expression of the transcripts is shown as the percentage of 0 hour values.

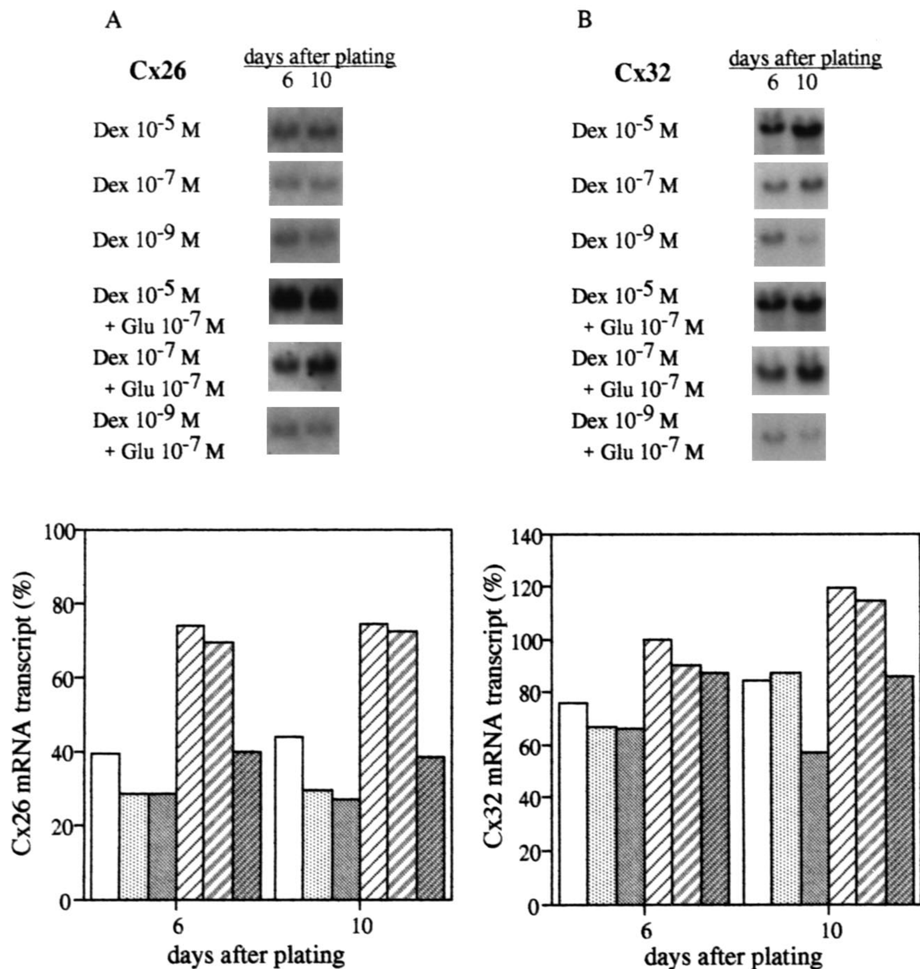

Fig. 5. Effects of the concentration of dexamethasone with or without 10^{-7} M glucagon on Cx26 (A) and Cx32 mRNA (B) transcripts. The medium was changed to 2% DMSO medium with various concentrations of dexamethasone added, with or without 10^{-7} M glucagon from 96 hours. Dex: dexamethasone, Glu: glucagon. The results are shown in the histograms (A,B); □: with 10^{-5} M dexamethasone, ▥: with 10^{-7} M dexamethasone, ▦: with 10^{-9} M dexamethasone, ▨: with a combination of 10^{-5} M dexamethasone and 10^{-7} M glucagon, ▨: with a combination of 10^{-7} M dexamethasone and 10^{-7} M glucagon, ▨: with a combination of 10^{-9} M dexamethasone and 10^{-7} M glucagon. Expression of the transcripts is shown as the percentage of 0 hour values.

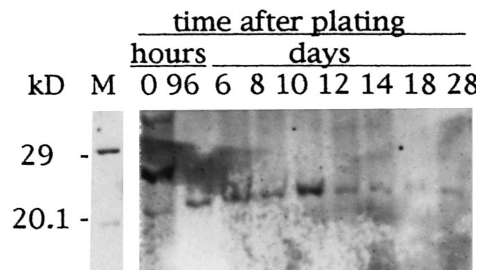

Fig. 6. Western blot analysis of primary cultured rat hepatocytes for Cx26. The medium was changed to 2% DMSO medium with 10^{-7} M glucagon from 96 hours. NaOH-insoluble fractions were separated by electrophoresis in a 12.5% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. After transfer, the blots were stained with antibodies against Cx26 and detected by the enhanced chemiluminescence (ECL) Western blotting system. The blots were exposed to film for 3 minutes. M: marker.

influenced by the concentrations of dexamethasone just as that of Cx26 mRNA (Fig. 5B).

Induction of Cx26 protein by glucagon

Fig. 6 shows the changes in the amount of the Cx26 protein in the hepatocytes. In this case 2% DMSO and 10^{-7} M glucagon were added to the medium from 96 hours. At day 10, the highest amount of Cx26 protein was observed and the expression was maintained until day 28. On the other hand, without glucagon, the expression of Cx26 protein in the cells was low (data not shown).

Effects of glucagon, dib-cAMP and dexamethasone on the localization of Cx26 and Cx32 proteins

The morphology of the hepatocytes treated with 10^{-7} M glucagon from 96 hours is shown in Fig. 7. At day 6 (Fig. 7d), bile canaliculi-like structures, which were not observed at 96 hours (Fig. 7a), were partly formed. However, with time in culture, the structures disappeared again and the borders of the cells became clear (Fig. 7g and j). Fluorescent immunocytochemistry was carried out to examine the Cx26- and Cx32-immunoreactivity of the hepatocytes from 8 hours to day 24. Cx26-positive macular spots were observed until 8 hours after plating, and thereafter they were not found in the hepatocytes (Fig. 7b). However, when the cells were treated with 2% DMSO and 10^{-7} M glucagon from 96 hours, Cx26-positive spots partly reappeared between adjacent cells observed near the bile canaliculi-like structures (Fig. 7e). The number of positive spots gradually increased together with the time of DMSO treatment and finally, most cells possessed positive spots (Fig. 7h and k). On the other hand, Cx32-positive spots randomly reappeared between adjacent hepatocytes as previously reported (Kojima et al., 1995). With time in culture, thin Cx32-immunoreactive lines partly surrounding hepatocytes were also seen and small dots were observed in the cytoplasm of the hepatocytes (Fig. 7i and l). Eventually even at day 24, both Cx26- and Cx32-positive spots could be observed (Fig. 7k and l). To examine the localizations of Cx26- and Cx32-positive spots which were observed between adjacent cells, we performed double-staining for Cx26 and Cx32 and visualized the result with a confocal lazer microscope. In some of the cells at day 10, Cx26- and Cx32-positive spots were observed in the same plaques (Fig. 8a,b and c). However, in most of the cells, Cx32-positive spots were observed much more than Cx26-positive spots and many single spots of Cx32 were oberved (data not shown). We found that 0.5 mM dib-cAMP could induce Cx26- and Cx32-immunoreactivity between adjacent hepatocytes in as many cells as in the treatment with 10^{-7} M glucagon (data not shown). Hepatocytes cultured in the medium with 10^{-9} M dexamethasone did not show Cx26- and Cx32-immunoreactivities (data not shown).

Effect of glucagon and dexamethasone on GJIC

As we recently reported (Kojima et al., 1995), the hepatocytes were well maintained in the medium with 2% DMSO and Cx32 was well expressed together with extensive GJIC. To examine the effect of glucagon and dexamethasone on GJIC, scrape-loading/dye transfer was performed in the hepatocytes treated with 10^{-7} M glucagon and dexamethasone (10^{-5}, 10^{-7}, 10^{-9} M) at day 10. The morphology of the hepatocytes is shown in Fig. 9a, c and e. When the cells were cultured in the medium supplemented with more than 10^{-7} M dexamethasone, the addition of glucagon did not affect the dye spread (Fig. 9b and d). However, when the cells were cultured with 10^{-9} M dexamethasone, the dye spread was markedly suppressed even if glucagon was added to the medium (Fig. 9f).

DISCUSSION

We recently showed that Cx32 in primary rat hepatocytes was well reexpressed and maintained for a long time in the modified L-15 medium supplemented with EGF and 2% DMSO (Kojima et al., 1995). However, in that study, the expression of Cx26 was very low and unstable compared to Cx32. In the present study, we demonstrated that Cx26 could be dramatically induced by the addition of glucagon. Expression of mRNA as well as protein of Cx26 was shown in primary hepatocytes. In addition, to induce Cx26 mRNA and functionally active gap junctions, the existence of dexamethasone in the medium was shown to be very important.

It has been reported that in vitro cAMP increases junctional conductance in a variety of cell types (Piccolino et al., 1984; McMahon et al., 1989). In primary rat hepatocytes, cAMP was also shown to delay the disappearance of gap junctions by increasing the stability of Cx32 mRNA (Sáez et al., 1989b). The administration of glucagon caused a rapid recovery of coupling of dissociated rat hepatocytes accompanying an increase of cytoplasmic cAMP (Sáez et al., 1990). Miller et al. (1988) demonstrated that a genomic clone of Cx32 possessed the cAMP response element sequence. These results showed that the expression of Cx32 might be transcriptionally and post-transcriptionally regulated by cAMP. However, the induction and the regulation of Cx26 in hepatocytes have not been well investigated, although the sequence of Cx26 cDNA was reported (Zhang and Nicholson, 1989). In the present experiment, we showed that Cx26 mRNA could be regulated by glucagon and cAMP. This result suggests that the expression of Cx26 may be tran-

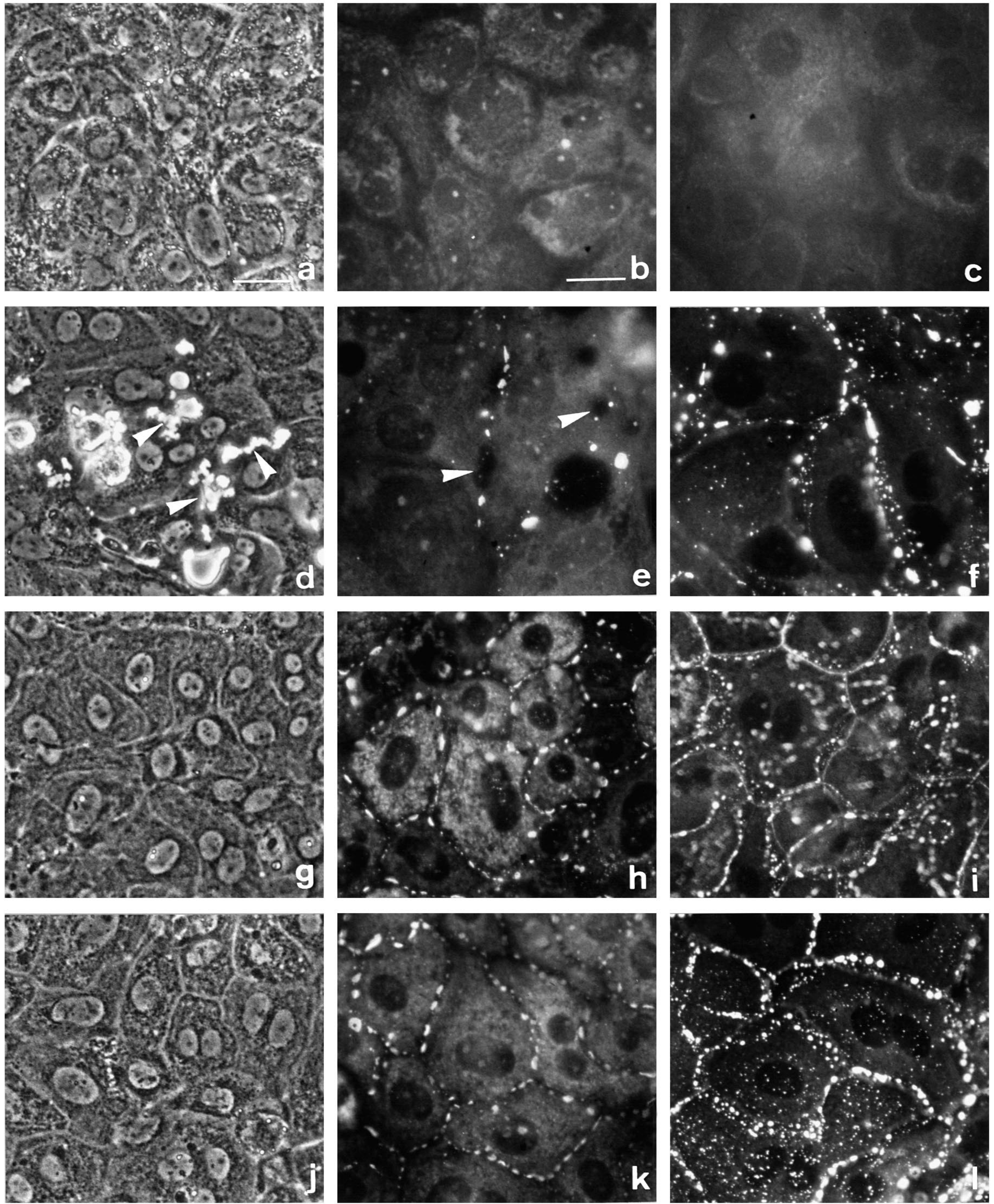

Fig. 7. Photographs of cells (a,d,g,j) and fluorescent immunocytochemistry of Cx26 (b,e,h,k) and Cx32 (c,f,i,l) in primary cultured rat hepatocytes. (a,b,c) At 96 hours after plating. Then, the cells were cultured in 2% DMSO medium with 10^{-7} M glucagon. (d,e,f) Day 6 (2 days after treatment). Arrowheads show bile canaliculi-like structures. (g,h,i) Day 14. (j,k,l) Day 24. Bars: 20 µm for a,d,g and j; 10 µm for b,c,e,f,h,i,k and l.

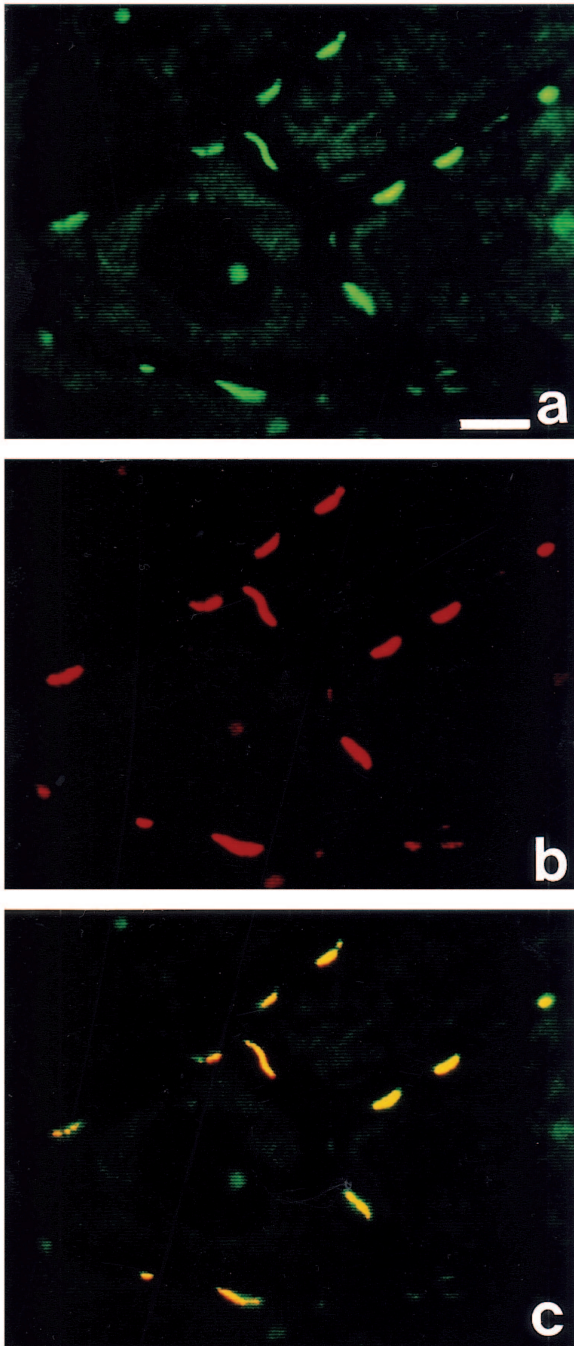

Fig. 8. Double fluorescence immunocytochemistry of Cx26 and Cx32 in hepatocytes cultured in 2% DMSO medium with 10^{-7} M glucagon at day 10. (a) Cx26-positive spots are observed green by FITC. (b) Cx32-positive spots are observed red by horse Texas Red. (c) Co-localization of Cx26- and Cx32-positive spots are observed yellow. Bar, 5 μm.

scriptionally regulated and that there is a possibility of the existence of a cAMP response element in the genome. Futhermore, the induction of Cx26 by glucagon was much more marked than that of Cx32 in this experiment. Without glucagon, expression of Cx32 mRNA and protein was easily detected and the expression was stable, while expression of Cx26 mRNA and protein was very low and unstable. These results suggested that the expression of Cx26 may be more sensitive to glucagon than that of Cx32.

Glucocorticoids such as dexamethasone have also been shown to modulate GJIC in hepatocytes (Klauning et al., 1990; Stutenkemper et al., 1992). Recently, Kwiatkowski et al. (1994) reported that in primary rat hepatocytes, dexamethasone maintained GJIC and Cxs mRNA. In the present study, the existence of dexamethasone in the medium was also necessary to maintain GJIC and Cxs mRNA. In addition, for induction of Cx26 mRNA, the existence of more than 10^{-7} M dexamethasone in the medium was shown to be very important.

In the adult rat liver, Cx26 is preferentially localized in hepatocytes of periportal zones, whereas Cx32 is uniformly distributed through the lobules (Sakamoto et al., 1992; Traub et al., 1989). Recently, Kojima et al. (1994) reported that, in female rat livers, the distribution of Cx26 rapidly changed after perfusion with modified medium. In those studies, Cx26 was uniformly distributed through the lobules within 30 minutes. The phenomenon was not accompanied by the increase of the amounts of both mRNA and protein. This means that post-translational regulation of Cx26 also exists in the liver. In addition, we showed that estrogen influenced the distribution of Cx26 in the liver. However, Cx32 did not show any change with perfusion and estrogen. On the other hand, the gradient of glucagon receptors (GRs) is known in adult rat liver lobules (Berthoud et al., 1992). Hepatocytes in pericentral zones express many more GRs than those in periportal zones because of down-regulation of the receptors induced by a higher concentration of glucagon in the periportal zone than in the pericentral zone. In the present experiment, expression of Cx26 could be induced by addition of glucagon to the primary cultures and, with time in culture, Cx26-positive spots could be observed between most adjacent hepatocytes isolated from both the pericentral zone and the periportal zone. In the adult rat liver, the expression of Cx26 may also be influenced by the concentration of intercellular glucagon within the acinar lobules. Futhermore, Cx32 may be more conservative than Cx26, and Cx26 may be easily influenced by some factors, especially hormones (Stutenkemper et al., 1992).

In the present experiment, after the treatment with glucagon or dib-cAMP, bile canaliculi-like structures were often observed between adjacent hepatocytes. Interestingly, Cx26-immunoreactivity initially appeared between adjacent hepatocytes near the structures, while Cx32 randomly appeared between adjacent cells. Bile flow formation in the bile canaliculus is thought to need organized and periodic contraction of bile canaliculi by cell-cell transduction of contraction signals through GJs (Watanabe et al., 1991). Thus, the initial appearance of Cx26 near the bile canaliculi-like structures suggests that Cx26 may be related to the organization of contraction of bile canaliculi.

Although we speculate that Cx26 in the liver might be regulated by glucagon and be related to bile canalicular contraction, the real functions of Cx26 are still unknown. In fact, in the present experiment, the ability of GJIC measured by the scrape-loading dye transfer method did not show a significant difference between the hepatocytes highly expressing Cx32 without glucagon and the cells highly expressing both Cx26 and Cx32 with glucagon (Fig. 9). Thus, we need to further examine the roles of Cxs in the liver.

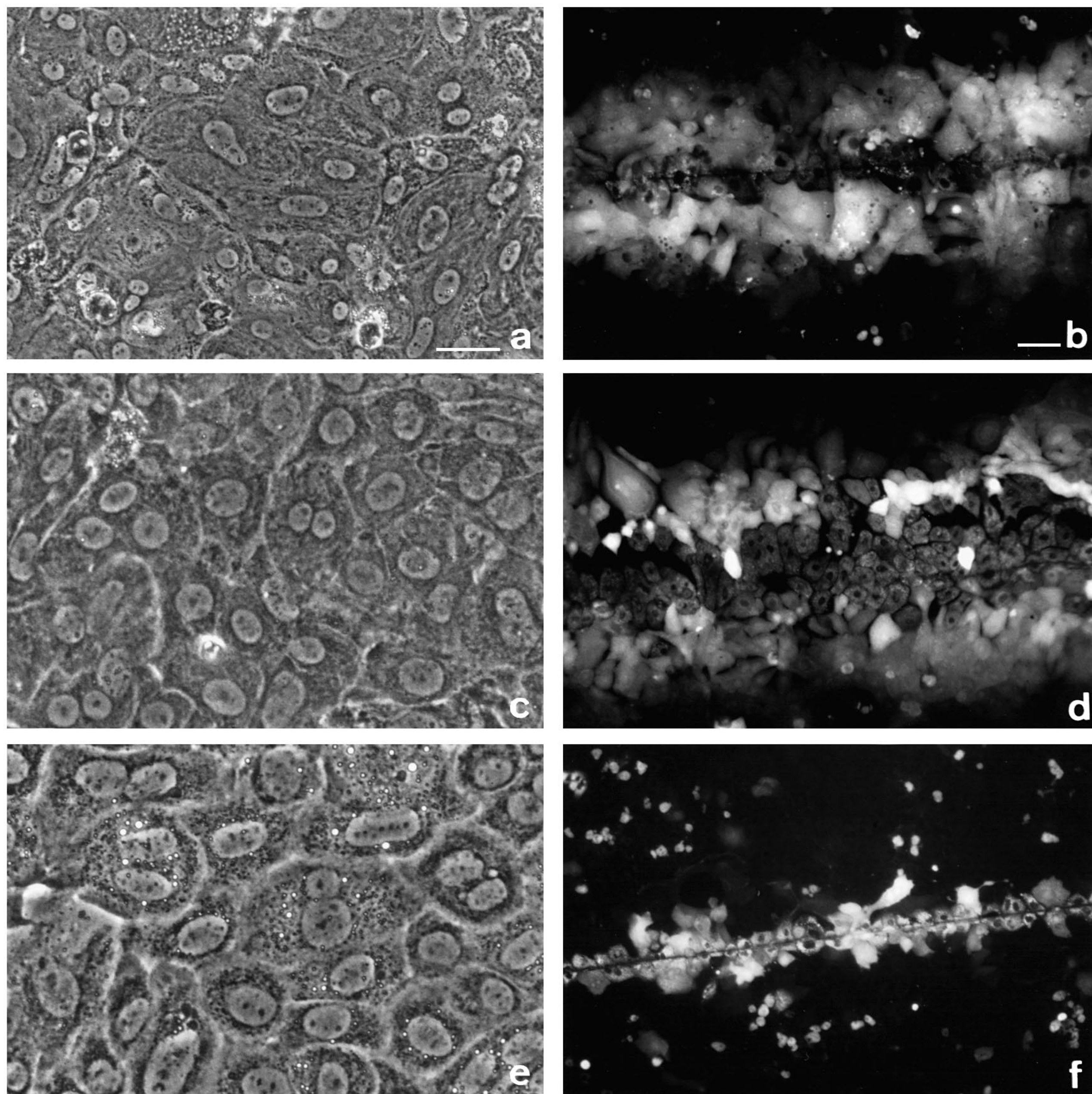

Fig. 9. Lucifer Yellow distribution in primary cultured rat hepatocytes at day 10. The cells were cultured in the medium supplemented with 2% DMSO and 10^{-7} M dexamethasone (a,b). The cells were cultured in the medium supplemented with 2% DMSO, 10^{-7} M dexamethasone and 10^{-7} M glucagon (c,d). The cells were cultured in the medium supplemented with 2% DMSO, 10^{-9} M dexamethasone and 10^{-7} M glucagon (e,f). (a,c,e) Phase-contrast photographs of the cells. (b,d,f) Lucifer Yellow distribution in the hepatocytes was measured using the scrape-loading technique as described in Materials and Methods. Bright cytoplasms of the cells show that the dye spreads into the adjacent cells. Bars: 20 µm for a,c and e; 80 µm for b,d and f.

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