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Importance du stade du cycle cellulaire sur les embryons reconstitués par transfert nucléaire

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Importance du stade du cycle cellulaire sur les embryons reconstitués par transfert nucléaire

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Le taux de développement des embryons produits par transfert nucléaire demeure encore très faible. Cela pourrait être dû à des incompatibilités qui s'expriment lors de l'interaction nucléocytoplasmique ou au remodelage incomplet des noyaux transplantés. Notre principal objectif avec ce travail était de vérifier si ces phénomènes étaient influencés par le stade du cycle cellulaire lors de la reconstitution des embryons.

Le premier projet visait à tester différents agents d'induction de l'activation des ovocytes bovins, c'est-à-dire des substances qui lèvent le blocage en métaphase de la méiose, en prévision du transfert nucléaire. Nous avons démontré que l'exposition à 7 % d'éthanol pendant 5 minutes induisait l'activation de 82 % des ovocytes bovins quand le traitement est effectué après 30 h de maturation *in vitro*. Les ovocytes activés selon ce protocole ont ensuite été utilisés pour établir de nouvelles méthodes de préparation des ovocytes hôtes en vue du clonage. Le taux de réussite de l'énucléation d'ovocytes hôtes en télophase 2 h après l'activation est de 98%, un taux significativement supérieur aux 59% obtenus lorsqu'on procède à l'énucléation avant l'activation. Par comparaison avec la méthode standard, la méthode d'énucléation au stade de télophase permet d'accroître le taux de développement et la qualité des embryons produits par transfert nucléaire. La supériorité de la méthode *télophase* pourrait s'expliquer par le fait qu'on sélectionne seulement les ovocytes qui ont répondu à l'activation, que l'énucléation est effectuée sans recours à des colorants ou à une exposition aux rayons UV et que le volume de cytoplasme prélevé lors de l'énucléation est faible.

Dans le deuxième projet, notre objectif était d'utiliser des spermatozoïdes traités aux ultraviolets pour induire l'activation des ovocytes bovins. Si cette procédure, utilisée avec succès chez les poissons et les amphibiens, s'avérait praticable chez les mammifères, elle

pourrait servir à l'activation des ovocytes non *vieillis*. Nous avons démontré que les spermatozoïdes exposés à 10 mJ/cm^2 peuvent activer une grande proportion des ovocytes, mais que le développement jusqu'au stade blastocyste était quasiment nul. Ceci semblait indiquer que la chromatine des spermatozoïdes était endommagée et que cette technique pourrait donc servir à l'activation des ovocytes hôtes aux fins de clonage. Les embryons reconstitués avec des ovocytes activés par des spermatozoïdes irradiés ont présenté un faible développement par comparaison avec les ovocytes activés par des spermatozoïdes non irradiés ou activés artificiellement. Des analyses de la chromatine effectuées après la première division indiquent que le blocage du développement est probablement imputable à des ruptures de la chromatine provoquées par l'irradiation. Le recours à cette méthode pour l'activation des ovocytes hôtes en vue du clonage doit donc être écarté.

Avec le troisième projet, nous voulions vérifier si l'histone somatique H1 était remodelable après le transfert nucléaire et déterminer si le moment choisi pour l'activation des ovocytes hôtes pouvait intervenir sur ce remodelage. À l'aide d'un anticorps qui se lie spécifiquement à l'histone somatique H1, nous avons démontré que cette protéine est remodelée après le transfert nucléaire c'est-à-dire qu'elle perd l'immunoréactivité au cours du premier cycle cellulaire. Le remodelage a été plus rapide dans les noyaux transplantés dans des ovocytes hôtes autour du moment de l'activation. Aucune corrélation n'a été observée entre la perte de l'immunoréactivité de l'histone H1 et la croissance des noyaux transplantés. L'inhibition de la réplication et de la transcription de la chromatine n'a pas eu d'impact sur le temps nécessaire au remodelage de l'histone H1. Cependant, l'inhibition de la synthèse des protéines et de l'activité kinase ont retardé le remodelage. L'effet de ces agents était beaucoup moins évident lorsque les noyaux transplantés provenaient de blastomère au stade G1. Le stade cellulaire des noyaux

transplantés serait donc aussi important dans le remodelage de la chromatine transplantée. Indépendamment du type d'ovocyte hôte utilisé, le réassemblage de l'histone somatique H1 dans la chromatine se déroule au stade 8 à 16 blastomères, soit au même stade que chez les embryons non manipulés. Il y a donc modification spécifique de la chromatine après le transfert nucléaire et ce phénomène pourrait jouer un rôle dans la réacquisition de la totipotence nucléaire.

La dernière étude visait à déterminer l'importance, chez la souris, du stade du cycle cellulaire des ovocytes hôtes et des blastomères donneurs sur le remodelage de l'histone somatique H1 dans la chromatine transplantée. Les ovocytes hôtes en métaphase ont remodelé l'histone H1 plus rapidement que les ovocytes en télophase (3 h après l'activation). Par contre, les ovocytes en interphase (8 h après l'activation) ont été incapables de remodeler l'histone H1 de la majorité des noyaux transplantés. La croissance des noyaux transplantés a aussi été influencée par le stade du cycle cellulaire des ovocytes hôtes. De la même façon que pour le remodelage de l'histone H1, la croissance nucléaire a été supérieure dans les ovocytes hôtes en métaphase et en télophase par comparaison avec les ovocytes en interphase. La formation d'un pronoyau dans les ovocytes hôtes avant le transfert nucléaire est responsable, du moins en partie, de la réduction de la capacité des ovocytes en interphase à remodeler la chromatine transplantée (DiBerardino et Hoffner, 1975). Le stade du cycle cellulaire des noyaux transplantés a également influencé le temps requis pour le remodelage de l'histone H1. L'histone H1 a perdu plus rapidement son immunoréactivité chez les noyaux transplantés au stade de métaphase que chez les noyaux au stade G1/S ou S/G2. La fusion de blastomères de souris avec des ovocytes bovins a permis de montrer que les mécanismes de commande du remodelage de l'histone somatique H1

fonctionnent entre différentes espèces et sont influencés par le stade du cycle cellulaire des ovocytes hôtes.

L'ensemble de nos résultats montre clairement que le remodelage des noyaux transplantés est influencé par le stade du cycle cellulaire au moment de la reconstitution des embryons. Ceci pourrait non seulement avoir d'importantes répercussions dans le domaine de la production des embryons clonés, mais aussi contribuer à élucider les mécanismes de commande de la réacquisition de la totipotence.

Mots clés : Transfert nucléaire, cycle cellulaire, ovocytes, embryons, énucléation, activation, spermatozoïdes, radiation ultraviolette, fécondation, histone H1, chromatine, reprogrammation nucléaire, bovins, souris.

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LISTE DES ABRÉVIATIONS

BrdU - 5-bromo-2'-deoxyuridine 5'-triphosphate.

CA – Courant alternatif.

CD – Courant direct.

CDK – Kinases cyclines-dépendantes.

CG – Cellules germinales.

CPC – Condensation prématurée de la chromatine.

CSF – Facteur cytostatique.

FIV - Fécondation in vitro.

G0 - Stade de quiescence du cycle cellulaire avant le début de la phase S.

G1 – Stade du cycle cellulaire situé entre la division et le début de la synthèse de l'ADN (phase G1).

G2 – Stade du cycle cellulaire situé entre la fin de la réplication de l'ADN et la division (phase G2).

H1 – Histone H1.

M – Métaphase.

MAPK – Kinases activées par de mitogènes.

MI – Stade métaphase I de la méiose.

MII – Stade métaphase II de la méiose.

MIV - Maturation in vitro.

MPF – Facteur de promotion de la phase M.

RVG – Rupture de la vésicule germinative.

S – Stade du cycle cellulaire pendant lequel la réplication de l'ADN se déroule (phase S).

UV – Ultraviolet

VG – Vésicule germinative.

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DÉDICACE

À la mémoire de mon père,

À ma mère,

À Márcia, Pedro et Jean, avec
tout mon amour et tendresse.

I. INTRODUCTION

Initialement présentée comme un outil pour l'étude de l'équivalence nucléaire (Spemann, 1938), la technologie du transfert nucléaire présente en fait un remarquable intérêt pour la recherche fondamentale en biomédecine et les applications commerciales en biotechnologie des productions animales. En plus d'avoir permis de répondre à la question de l'équivalence nucléaire en démontrant que même les cellules animales adultes peuvent produire des animaux fertiles (Wilmut et al., 1997; Wakayama et al., 1998), le transfert nucléaire a rendu possible d'autres avancées notables. Mentionnons, entre autres, les études sur les interactions nucléocytoplasmiques (McGrath et Solter, 1984b; Smith et al., 1988; Szöllösi et al., 1988; Campbell et al., 1993), sur l'importance du génome mâle et femelle, dont l'effet des empreintes géniques (*imprinted genes*), sur le développement embryonnaire (Surani et al., 1984; McGrath et Solter, 1984a; Kono et al., 1996b) et sur les mécanismes de transmission du génome mitochondrial (Meirelles et Smith, 1997; Meirelles et Smith, 1998). Par ailleurs, la principale application du transfert nucléaire est la production d'animaux transgéniques (Schnieke et al., 1997; Stice et al., 1998; Cibelli et al., 1998) et l'amélioration génétique des troupeaux (Colleau, 1992).

Malgré les récents et remarquables progrès à ce chapitre, le taux de développement à terme des embryons produits par transfert nucléaire demeure encore très faible (Kruip et den Daas, 1997). Des études supplémentaires seront donc nécessaires pour améliorer cette technologie de manière à obtenir des résultats uniformes et satisfaisants. La réponse à ce problème viendra probablement d'une meilleure compréhension des facteurs qui commandent les interactions nucléocytoplasmiques lors de la reconstitution des embryons. Plusieurs études suggèrent que ces interactions peuvent être influencées par le stade du cycle cellulaire (revue par Campbell et al., 1996a; Fulka, Jr. et al., 1996). En effet, les travaux classiques de Johnson et Rao (1970) et Rao et Johnson (1970)

ont montré que, lors de la fusion de cellules somatiques, le cycle cellulaire joue un rôle important dans l'activité et la morphologie de la chromatine. Par la suite, l'utilisation du transfert nucléaire a permis de démontrer l'importance du cycle cellulaire dans la croissance nucléaire (Czolowska et al., 1984; Szöllösi et al., 1988; Collas et Robl, 1991), l'activité de la chromatine (Barnes et al., 1993; Borsuk et al., 1996; Smith et al., 1996) et le développement des embryons reconstitués (Smith et al., 1988; Collas et Robl, 1991; Collas et al., 1992b; Campbell et al., 1994). En outre, l'association de stades spécifiques du cycle cellulaire serait peut-être un des principaux facteurs responsables de la réussite du clonage à partir de cellules d'animaux adultes (Wilmut et al., 1997; Wakayama et al., 1998). Si l'importance de maîtriser le cycle cellulaire avant la reconstitution des embryons fait peu de doute, plusieurs questions subsistent. Ainsi, nous ne savons pas encore si le cycle cellulaire affecte le remodelage des composants structuraux de la chromatine lors du transfert nucléaire.

Parmi les autres facteurs susceptibles d'intervenir dans le développement des embryons reconstitués par transfert nucléaire, on peut penser à certaines limites de la technique, dont l'utilisation de l'ultraviolet pour la préparation des ovocytes hôtes (Smith, 1992b) et le recours à des ovocytes hôtes *vieillis* pour faciliter l'activation (Heyman et al., 1994; Stice et al., 1994).

L'objectif des études dont fait état cette thèse était d'abord de vérifier si la manipulation du cycle cellulaire des ovocytes hôtes permettrait de mettre au point des méthodes de clonage plus efficaces puis d'étudier l'impact du cycle cellulaire sur le remodelage des noyaux transplantés.

II. REVUE DE LA LITTÉRATURE

1. Le transfert nucléaire

Le clonage d'animaux adultes par transfert de noyaux de cellules somatiques dans des ovocytes énucléés est une des avancées scientifiques récentes les plus spectaculaires (Wilmut et al., 1997; Wakayama et al., 1998). C'est la somme des connaissances fondamentales accumulées au cours des années dans le cadre d'études sur le transfert nucléaire qui a rendu possible cette réussite. Cependant, plusieurs questions demeurent sans réponse en ce qui a trait aux mécanismes en jeu dans le transfert nucléaire. Une meilleure compréhension des mécanismes qui commandent les interactions noyau/cytoplasme et de leurs effets sur le développement des embryons reconstitués pourrait être déterminante pour l'obtention de résultats plus uniformes.

1.1. Historique

Le transfert nucléaire a d'abord été proposé par Spemann (1938) pour trancher la question de l'équivalence nucléaire, c'est-à-dire la modification ou non du noyau pendant la différenciation cellulaire au cours du développement. Il a fallu attendre jusqu'en 1952 les premiers résultats publiés (Briggs et King, 1952). Les auteurs rapportaient que, chez la grenouille, des noyaux prélevés d'embryons au stade blastula puis transférés dans des ovocytes énucléés permettaient la reprise du développement embryonnaire jusqu'à terme. Ces noyaux étaient donc fonctionnellement équivalents à des noyaux de zygotes. Ces observations ont servi de base à l'élaboration des concepts actuels d'équivalence nucléaire, soit que les noyaux transférés dans des ovocytes énucléés capables de reprendre leur développement et de le poursuivre à terme sont dits totipotents. Plusieurs autres études menées chez les amphibiens ont confirmé la totipotence des noyaux embryonnaires (article de revue DiBerardino, 1980; Gurdon, 1986). Il s'est avéré que les cellules

intestinales de grenouille au stade larvaire l'étaient également (Gurdon et Uehlinger, 1966). Cependant, les cellules animales d'adulte ne peuvent produire d'animaux adultes même si, par ailleurs, elles peuvent provoquer la reprise du développement embryonnaire (Gurdon et al., 1975). Il semblerait donc que la différentiation qui survient dans les cellules animales adultes rend la chromatine incapable de repasser par toutes les étapes du développement.

Les premières naissances de mammifères produits par transfert nucléaire ont été rapportées par Illmensee et Hoppe (1981). Ces chercheurs ont réussi à produire des souris par transfert de noyaux provenant du bouton embryonnaire. D'autres groupes de recherche incapables de reproduire ces résultats en déduisaient que les noyaux originaires d'embryons de plus de 2 blastomères ne permettraient pas le développement à terme (McGrath et Solter, 1984b). Pourtant, deux ans plus tard, Willadsen (1986) rapportait la naissance d'agneaux à la suite du transfert de noyaux provenant d'embryons de 8 à 16 blastomères. Chez les mammifères domestiques comme chez les amphibiens, les noyaux ayant acquis une activité transcriptionelle demeurent donc totipotents. Ces résultats ont ensuite été reproduits chez d'autres espèces, dont le bovin (Prather et al., 1987), le lapin (Stice et Robl, 1988) et le porc (Prather et al., 1989). Des études subséquentes ont démontré que même les cellules de bouton embryonnaire pouvaient soutenir le développement à terme (Smith et Wilmut, 1989; Keefer et al., 1994). Finalement, Campbell et ses collaborateurs (1996b) ont réussi à produire des agneaux par transfert de noyaux de cellules de lignées embryonnaires cultivées et soumises à plusieurs passages *in vitro*. Or, la longue mise en culture et les changements morphologiques de ces cellules les excluaient de la catégorie embryonnaire : les cellules somatiques étaient-elles aussi totipotentes? Cette question a reçu une première réponse lorsque Wilmut et ses collaborateurs (1997) ont annoncé la naissance d'animaux à la

suite de transferts nucléaires à partir de cellules mammaires en culture. Plus récemment, d'autres chercheurs ont démontré que même des cellules ovariennes et des oviductes peuvent soutenir le développement à terme et donc sont totipotentes (Wakayama et al., 1998; Kato et al., 1998).

Les procédures actuelles de transfert nucléaire chez les mammifères sont issues de la technique décrite par Willadsen (1986). Elles sont représentées schématiquement à la figure 1.

1.2. Préparation des noyaux à transférer

Des cellules de différents tissus ont déjà été utilisées avec succès comme donneuses de noyaux pour le transfert nucléaire chez les mammifères. On a eu recours, entre autres, à des embryons à différents stades du développement pré-implantation (McGrath et Solter, 1984b; Willadsen, 1986; Smith et Wilmut, 1989), à des lignées cellulaires embryonnaires ou cellules souches (Campbell et al., 1996b; Wells et al., 1997), à des lignées cellulaires foetales (Cibelli et al., 1998), à des cellules de tissus adultes cultivées *in vitro* (Wilmut et al., 1997) et à des cellules prélevées directement d'animaux adultes (Wakayama et al., 1998; Kato et al., 1998).

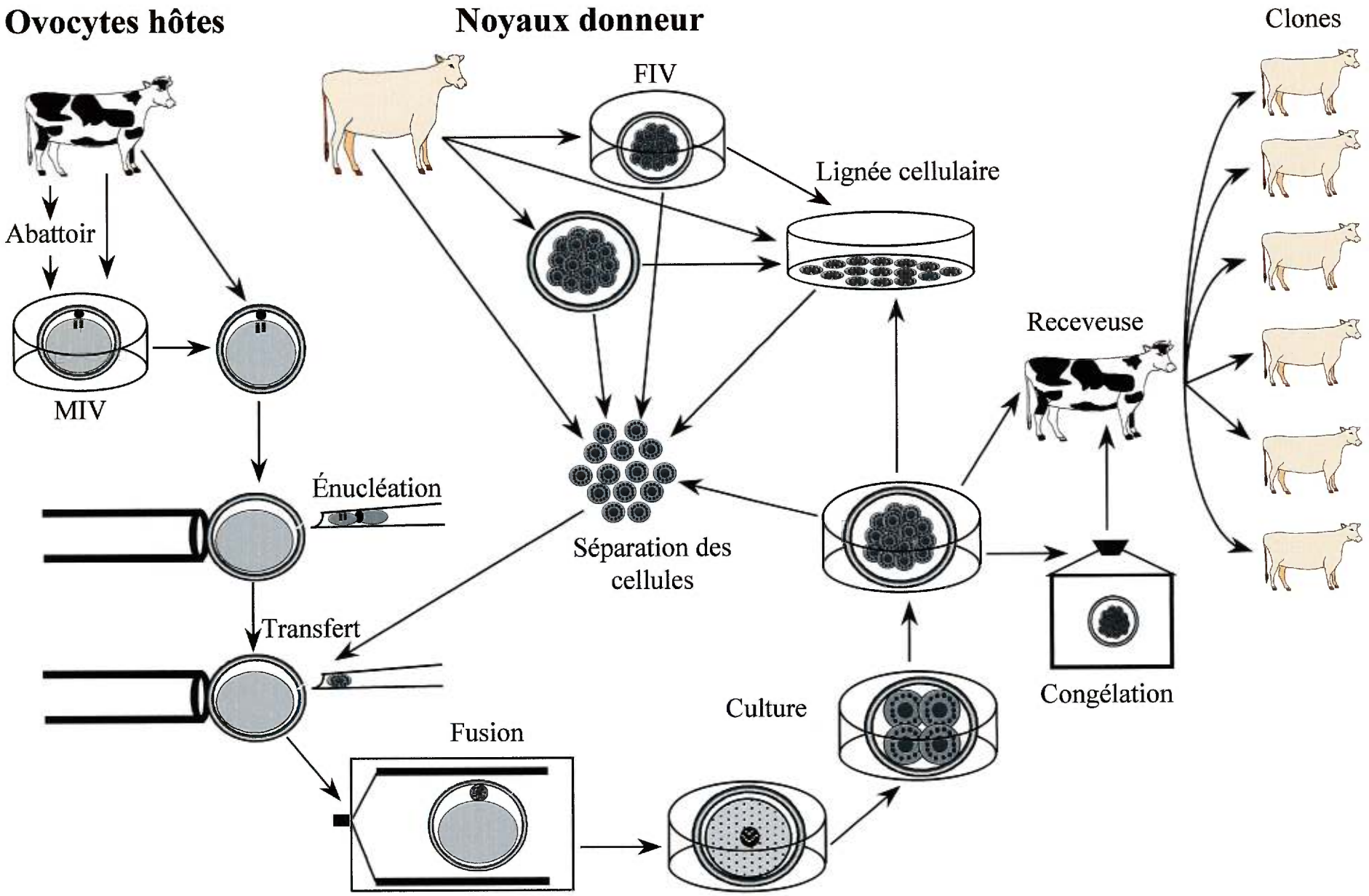
Ovocytes hôtes
Noyaux donneur
Clones
FIV
Lignée cellulaire
Abattoir
MIV
Receveuse
Énucléation
Séparation des cellules
Transfert
Culture
Congélation
Fusion

Figure 1.

Schéma de la technique de clonage normalement utilisée chez les mammifères (adaptée de Smith, 1992a). Les ovocytes hôtes peuvent être récoltés chez des femelles vivantes ou récupérés dans des ovaires provenant de l'abattoir puis mûris *in vitro*. Les noyaux peuvent être issus d'embryons, de fœtus ou même de tissus d'animaux adultes. Les embryons reconstitués sont cultivés *in vitro* jusqu'au stade de morula/blastocyste. Ils peuvent alors être reclonés, transférés dans une receveuse, congelés ou même utilisés pour établir des lignées de cellules souches.

Quand les noyaux proviennent de zygotes ou d'embryons ayant subi 1 ou 2 divisions, le prélèvement se fait par microchirurgie. Les embryons donneurs sont incubés en présence de cytochalasine, un déstabilisateur des microfilaments, ce qui permet d'aspirer le noyau et une portion du cytoplasme environnant (caryoplaste) sans lyser la membrane plasmique. Par ailleurs, si les noyaux proviennent d'un embryon plus développé, on utilise toute la cellule donneuse. La zone pellucide doit alors être retirée par digestion avec des protéases ou une solution de pH acide ou encore par micromanipulation ceci afin de séparer les cellules. La masse cellulaire est aspirée à plusieurs reprises dans une fine pipette de manière à séparer les blastomères. Si les embryons en sont déjà au stade compact, la désagrégation des blastomères sera facilitée par l'incubation dans une solution sans calcium et sans magnésium. Quant aux cellules du bouton embryonnaire, elles sont désagrégées à l'aide d'une solution de trypsine une fois le bouton séparé par immunochirurgie. Les cellules provenant de lignées en culture sont aussi isolées à l'aide de la trypsine.

Une fois les cellules ou le caryoplaste isolés, ils sont insérés dans l'espace périvitellin des ovocytes hôtes au préalable énucléés. L'incorporation du noyau dans l'ovocyte est induite par l'électrofusion de la membrane plasmatique des cellules et des ovocytes. Plus récemment, Wakayama et collaborateurs (1998) ont modifié les procédures décrites initialement par Illmensee et Hoppe (1981). Leur technique consiste à lyser les cellules par succion dans une pipette mince puis d'appliquer à plusieurs reprises le même procédé aux noyaux afin de les débarrasser des débris de cytoplasme. La même pipette sert ensuite à percer la membrane cytoplasmique de l'ovocyte hôte pour enfin injecter le noyau directement dans le cytoplasme. Cette méthode semble avoir des avantages par rapport à l'électrofusion : entre autres, elle n'induit pas l'activation des ovocytes hôtes et l'injection d'un noyau exempt de

cytoplasme peut favoriser l'interaction du noyau transplanté avec le cytoplasme hôte. Ces modifications de la technique permettent peut-être un meilleur déroulement du remodelage nucléaire, qui est nécessaire pour réacquérir la compétence pour le développement.

1.3. Préparation des ovocytes hôtes

La préparation des ovocytes hôtes comprend plusieurs étapes, soit la récolte, l'énucléation, la reconstruction et l'activation. La récolte se fait différemment selon l'espèce. Chez les animaux de laboratoire, les ovocytes hôtes sont habituellement produits *in vivo* par un traitement de surovulation puis recueillis après l'euthanasie des animaux. Chez les bovins, ils sont plus souvent récupérés à l'abattoir puis mûris *in vitro*.

Les ovocytes doivent être libérés des cellules du cumulus avant d'être micromanipulés. Le plus souvent, ce sera obtenu par un traitement avec une solution de hyaluronidase (0,1% p/v) et le passage à travers une pipette mince ou l'agitation (vortex). L'énucléation se fait par microchirurgie, soit par aspiration de la chromatine et d'une portion du cytoplasme environnant. Ces micromanipulations sont effectuées dans une solution contenant de la cytochalasine afin d'éviter la lyse des ovocytes. Le choix de la procédure se fait en fonction de l'espèce et du stade cellulaire dans lequel se trouvent les ovocytes hôtes.

Pour les espèces comme le bovin, le porc et l'ovin, dont le cytoplasme renferme de nombreuses vésicules de lipides, seul l'emploi de colorants fluorescents comme la bis-benzamide (Hoechst 33342 ou 33358) et l'exposition aux ultraviolets permettent la visualisation de la chromatine. Pour éviter de trop exposer les ovocytes à l'irradiation, le plus souvent on aspirera le cytoplasme autour du point d'extrusion du

premier globule polaire puis on exposera brièvement l'ovocyte aux ultraviolets afin de s'assurer que toute la chromatine a été éliminée. Même si des études suggèrent le contraire, l'utilisation de colorants fluorescents combinée à l'exposition aux ultraviolets peut avoir un effet délétère sur les organelles des ovocytes hôtes et compromettre la viabilité des embryons reconstitués (Smith, 1993). Chez d'autres espèces comme le lapin, le rat et la souris, la chromatine est visible au microscope optique à contraste de phase ou à contraste d'interférence surtout si l'énucléation se fait pendant le stade du pronucléus. La coloration de la chromatine n'est donc pas nécessaire dans ce cas.

Outre la microchirurgie, d'autres techniques d'énucléation ont été décrites : l'énucléation chimique (Fulka, Jr. et Moor, 1993), l'irradiation (Tsunoda et al., 1988; Yang et al., 1990; Bradshaw et al., 1995) ou la centrifugation (Tatham et al., 1995). Elles ont l'avantage de limiter les pertes de cytoplasme des ovocytes hôtes, mais elles n'ont pas été encore totalement validées.

On vient de le mentionner, le volume de cytoplasme éliminé lors de la préparation des ovocytes hôtes semble être un point important. En effet, la réduction du volume affecte le potentiel de développement et la qualité des embryons reconstitués (Evsikov et al., 1990; Westhusin et al., 1996; Zakhartchenko et al., 1997). Une technique d'énucléation qui tout en limitant la perte de cytoplasme ne nécessiterait ni colorants, ni irradiation pour la visualisation de la chromatine permettrait sans doute d'améliorer le développement des embryons produits par transfert nucléaire.

1.4. La fusion noyau/cytoplasme

L'induction de la fusion entre la membrane plasmique des cellules donneuses de noyaux et celle de l'ovocyte hôte est, à ce jour, la méthode plus efficace pour reconstituer des embryons.

Des études chez la souris ont montré que le virus Sendai inactivé induisait la fusion d'une grande proportion des embryons reconstitués (McGrath et Solter, 1983). Cette méthode, toujours utilisée de nos jours, est très efficace chez la souris (Smith et al., 1988; Smith et Wilmut, 1994; Kwon et Kono, 1996; Obata et al., 1998), mais moins chez d'autres espèces (Robl et al., 1992). Le polyéthylèneglycol et les liposomes utilisés pour la fusion des cellules somatiques (Wakahara, 1980; Spindle, 1981) ne semblent pas être appropriés pour induire la fusion lors de la reconstitution des embryons (Robl et al., 1992).

La technique la plus efficace chez de nombreuses espèces, dont la souris, le rat, le lapin, la brebis, le porc et le bovin (Robl et al., 1992), et donc la plus employée demeure l'électrofusion. Pour éviter la production et la dispersion de chaleur, qui pourrait endommager les cellules, l'application du courant électrique se fait dans une solution faiblement conductrice (Robl et al., 1992). Deux types de courant électrique sont normalement appliqués : le courant alternatif (CA), qui permet d'aligner la zone de contact de la membrane des cellules et des ovocytes en parallèle avec les électrodes, et le courant direct (CD), qui induit la fusion des membranes. Il est toutefois recommandé, surtout quand les cellules donneuses sont relativement petites par rapport aux ovocytes hôtes, de faire l'alignement manuellement. Bien que les paramètres d'intensité du CA n'aient pas été largement étudiés, il semble qu'une fréquence comprise entre 600 et 1000 kHz, un voltage de 5 à 6 V et une duré de 5 à

10 µsec soient efficaces. En ce qui a trait au CD, on parle d'un voltage de 0,6 à 3,6 kV/cm, d'une durée de 30 à 250 µsec et de 1 à 3 influx bien que, le plus souvent, on emploie un seul influx de 1,0 kV/cm pendant 60 µsec (Robl et al., 1992).

2. Facteurs biologiques en jeu dans le développement d'embryons reconstitués par transfert nucléaire

Les nombreuses études sur le transfert nucléaire menées chez différentes classes d'animaux ont permis d'identifier certains facteurs capables d'affecter le développement des embryons reconstitués: le degré de différenciation des noyaux (cellule donneuse), le stade cellulaire au moment de la reconstitution, les interactions noyau donneur/ovocyte hôte, et bien sûr, la qualité des noyaux donneurs et des ovocytes hôtes.

2.1. Le stade de différenciation des noyaux donneurs

Les études menées chez les amphibiens ont démontré que le stade de différenciation des noyaux à transférer affecte profondément le potentiel de développement des embryons reconstitués. En effet, l'utilisation de noyaux de cellules plus différenciées provoquait une réduction du développement des embryons reconstitués et une augmentation du nombre d'embryons anormaux (DiBerardino et Hoffner, 1970; DiBerardino, 1980; Gurdon, 1986; DiBerardino, 1987). Chez les mammifères, la plupart des observations faites jusqu'à présent portent sur le transfert de noyaux provenant d'embryons en tout début de développement. Les premières études réalisées avec des souris ont montré que le taux de développement des embryons reconstitués jusqu'au stade de blastocyste passe de 95% à 13% si on utilise des noyaux

provenant d'embryons au stade 1 ou 2 cellules et à 0% s'il s'agit d'embryons de 4 à 16 blastomères ou du bouton embryonnaire (McGrath et Solter, 1984b). Les modifications subies par la chromatine au moment de l'activation du génome embryonnaire, qui survient au stade de 2 blastomères chez la souris (Flach et al., 1982), rendrait donc la chromatine incapable de reprendre et de poursuivre le développement après le transfert nucléaire (Surani et al., 1987). Par contre, Cheong et ses collaborateurs (1993) ont démontré par la suite que des noyaux d'embryons de 4 à 8 blastomères possédaient ces compétences, quoique là encore dans une moindre mesure que les embryons plus jeunes. L'activation du génome ne serait donc pas la seule cause d'inhibition du développement. Chez d'autres espèces, dont les ovins (Willadsen, 1986; Smith et Wilmut, 1989), les bovins (Prather et al., 1987; Keefer et al., 1994), et les lapins (Collas et Robl, 1991), le taux de développement jusqu'au stade blastocyste est également tributaire du stade de différenciation des noyaux transplantés.

Bien que des comparaisons plus systématiques entre des cellules embryonnaires et somatiques n'aient pas encore été effectuées, des travaux réalisés avec des cellules fœtales ou d'animaux adultes ont démontré que le remodelage et le potentiel de développement après transfert nucléaire sont défavorisés par l'utilisation de noyaux plus différenciés (Tarkowski et Balakier, 1980; Tsunoda et al., 1989; Modlinski et al., 1990; Collas et Barnes, 1994; Ouhibi et al., 1996; Campbell et al., 1996b; Lavoir et al., 1997b; Wilmut et al., 1997b; Cibelli et al., 1998). Bien que d'autres facteurs puissent intervenir, on peut conclure de l'ensemble de ces études que le stade de différenciation de la chromatine a un impact certain sur le potentiel de développement des noyaux transplantés.

2.2. Le stade cellulaire des ovocytes hôtes

Les études antérieures, qui portaient avant tout sur l'effet des noyaux donneurs concluaient à un rôle de premier plan de la différenciation cellulaire. De nouvelles études suggèrent néanmoins que les ovocytes hôtes seraient probablement les grands responsables du remodelage de la chromatine, qui fait recouvrer à cette dernière sa totipotence après le transfert nucléaire. À cet égard, le stade cellulaire de l'ovocyte hôte au moment de la reconstitution semble être le facteur déterminant. Il faut toutefois nuancer cette affirmation puisque les résultats varient selon l'espèce et le stade de différenciation des noyaux donneurs. Les différences observées entre les espèces sont probablement reliées au moment de l'activation du génome embryonnaire. Lorsque l'activation débute pendant le premier cycle cellulaire comme chez la souris, le remodelage de la chromatine transplantée doit se faire plus rapidement que lorsqu'elle débute à partir du troisième cycle comme les bovins et les ovins. Par ailleurs, la chromatine des cellules plus différenciées doit subir un remodelage plus important.

Les premières indications de ce rôle déterminant de l'ovocyte hôte sont venues d'études chez la souris. Lorsque les ovocytes hôtes étaient au stade d'interphase du premier cycle cellulaire, les embryons reconstitués se développaient uniquement si les noyaux utilisés provenaient d'embryons qui n'avaient pas dépassé le deuxième cycle (McGrath et Solter, 1984b). Par contre, lorsqu'on utilise des ovocytes hôtes en métaphase ou récemment activés, le taux de développement après transfert de noyaux d'embryons de plus de 2 blastomères est élevé (Cheong et al., 1993; Otaegui et al., 1994). D'autres auteurs ont montré, toujours chez la souris, que le remodelage de la chromatine transférée, tel que mesuré à partir des modifications de la membrane nucléaire et de la croissance du noyau transféré, avait bien lieu lorsque

les ovocytes hôtes étaient en métaphase ou venaient d'être activés, mais pas si ces derniers était déjà en interphase (Czolowska et al., 1984; Szöllösi et al., 1988; Prather et al., 1991). Plus récemment, des auteurs ont rapporté un taux élevé de développement lors du transfert de noyaux d'embryons de 4 blastomères (Kwon et Kono, 1996) ou de cellules en phase finale de différenciation (Wakayama et al., 1998) dans des ovocytes hôtes en métaphase II activés plusieurs heures après le transfert. Le recours à des ovocytes hôtes en métaphase II comporte des avantages indéniables au regard du remodelage et du développement, mais il peut aussi entraîner des problèmes au chapitre de la morphologie de la chromatine surtout quand les noyaux transférés ne sont pas synchronisés à une phase spécifique du cycle cellulaire (Czolowska et al., 1992; Fulka, Jr. et al., 1993; Campbell et al., 1996a). Les difficultés liées à l'interaction noyau/cytoplasme seront discutées plus en détail dans les sections suivantes.

Chez les espèces où l'activation du génome embryonnaire débute au troisième cycle cellulaire comme les bovins, les ovins et les lapins (Telford et al., 1990), le taux de développement des embryons reconstitués à partir de noyaux d'embryons en pré-implantation est beaucoup plus élevé que chez la souris (Smith et Wilmut, 1989; Collas et Robl, 1991; Collas et al., 1992b; Campbell et al., 1994; Heyman et al., 1994; Stice et al., 1994; Zakhartchenko et al., 1995; Loi et al., 1998). Même si les ovocytes hôtes au stade de métaphase semblent induire un remodelage plus efficace des noyaux transférés chez ces espèces (Collas et Robl, 1991; Sun et Moor, 1991; Kanka et al., 1991; Smith et al., 1996), le taux de développement consécutif aux interactions noyau/ovocyte hôte est moins élevé qu'avec des ovocytes pré-activés (Barnes et al., 1993; Campbell et al., 1993; révisé par Campbell et al., 1996a). L'obtention d'ovocytes hôtes en interphase devient donc indispensable lorsque le transfert se fait à partir de cellules embryonnaires. Les facteurs en jeu

dans la régulation du cycle cellulaire des ovocytes hôtes et les techniques d'activation seront discutés à la section sur le cycle cellulaire.

Contrairement à la situation qui prévaut chez les cellules embryonnaires, jusqu'à présent, seuls les ovocytes hôtes en métaphase II permettent d'obtenir le développement à terme des embryons reconstitués à partir de noyaux de lignées cellulaires embryonnaires, fœtales ou adultes (Campbell et al., 1996b; Wells et al., 1997; Wilmut et al., 1997; Cibelli et al., 1998). Malgré le nombre limité d'études à ce jour et l'insuffisance des comparaisons avec les autres stades cellulaires des ovocytes hôtes, il semblerait que le stade de métaphase II est essentiel au remodelage des noyaux de cellules somatiques. Une bonne façon de valider cette hypothèse serait d'étudier l'impact du stade cellulaire de l'ovocyte hôte sur le remodelage de la chromatine transférée.

2.3. L'interaction noyau donneur/cytoplasme hôte

Il existe plusieurs indications que le devenir des noyaux transférés repose sur les interactions noyau donneur/ovocyte hôte au moment de la reconstitution (revue par Campbell et al., 1996a).

Les premières études faites chez la souris à l'aide d'embryons à différentes phases du premier ou deuxième cycle cellulaire ont montré que le développement des embryons reconstitués est altéré par l'asynchronisme entre noyau donneur et cytoplasme hôte (Smith et al., 1988; Smith et Wilmut, 1994). Il en est de même quand des noyaux à différents stades du deuxième et du quatrième cycle sont respectivement transférés dans des cytoplasmes à différents stades du premier et deuxième cycle (Smith et al., 1988; Smith et al., 1990; Smith et Wilmut, 1994). Ce phénomène pourrait peut-être s'expliquer par une perturbation

de certains événements cellulaires importants comme la réplication de l'ADN. Des études subséquentes chez le lapin (Collas et al., 1992b; Collas et al., 1992a) et la souris (Cheong et al., 1993; Otaegui et al., 1994) ont révélé que l'emploi de noyaux synchronisés au stade initial de l'interphase et d'ovocytes hôtes en métaphase réduit les dommages à la chromatine et améliore le taux de développement des embryons reconstitués.

Une des difficultés rencontrées avec les noyaux provenant d'embryons en pré-implantation est que la grande majorité des cellules se trouve en interphase et en processus de réplication de l'ADN (Barnes et al., 1993; Campbell et al., 1994). La synchronisation des cellules à un stade précis du cycle par l'emploi de substances de synchronisation de la division cellulaire pourrait s'avérer la solution à ce problème. Si cette procédure est efficace pour synchroniser les cellules embryonnaires chez certaines espèces comme le lapin (Collas et al., 1992b) et la souris (Samaké et Smith, 1996a), elle l'est moins chez d'autres comme le bovin (Samaké et Smith, 1996b; Samaké et Smith, 1997; Samaké et Smith, 1998).

À ce jour, on sait peu de choses sur les interactions noyau/cytoplasme qui se déroulent lors du transfert de noyaux de cellules somatiques. Il semblerait que la situation idéale au regard de l'interaction soit le transfert de noyaux sortis du cycle normal et entrés en G0 dans des ovocytes en métaphase (Campbell et al., 1996b; Wilmut et al., 1997).

Outre le stade cellulaire, d'autres facteurs cytoplasmiques peuvent avoir un effet sur les interactions noyau/cytoplasme. Les études de Meirelles et Smith (1998) montrent que le développement des embryons reconstitués par transfert des pronoyaux varie en fonction de la souche

de souris d'où provient le cytoplasme hôte : ce phénomène pourrait être imputable à l'incompatibilité des mitochondries.

3. Le cycle cellulaire, sa commande et ses effets sur le transfert nucléaire

Le cycle cellulaire est l'objet d'un grand intérêt de la part des biologistes depuis quelques années, un intérêt probablement relié aux travaux en oncologie. D'autres domaines ont bénéficié des nouvelles connaissances sur le cycle cellulaire, dont celui du transfert nucléaire. Des progrès ont certes été accomplis, mais certaines zones d'ombre demeurent (Sherr, 1996). Nous décrirons donc quelques aspects importants du cycle cellulaire et de son impact sur le transfert nucléaire.

3.1. Les phases du cycle cellulaire

Chez la plupart des cellules eucaryotes, le cycle est divisé en 4 phases distinctes. La phase G1 (de l'anglais gap = pause) représente une période de pause avant le début de la réplication de l'ADN, la phase S (synthèse), la période de réplication de l'ADN, la phase G2, une deuxième période de pause entre la phase S et le début de la division cellulaire, et, finalement, la phase M (mitose), la période de division cellulaire. Une série de mécanismes complexes et de points de contrôle, qui assurent la coordination entre les phase de croissance et de division et permettent d'éviter les chevauchements, sont responsables de la commande de la multiplication cellulaire. Trois points de vérification sont particulièrement importants : le point de contrôle G1 ou de départ situé entre la phase G1 et la phase S, le point de contrôle G2 ou d'entrée de la phase M situé à la fin de la phase G2 et qui amène les cellules en mitose

(phase M) et le point de contrôle de la métaphase ou de sortie de la phase M situé à la fin de la phase M et qui marque la fin de la division cellulaire (Alberts et al., 1994).

3.2. La commande du cycle cellulaire

Si plusieurs des facteurs en jeu dans la commande du cycle cellulaire sont connus, dont particulièrement ceux de l'entrée en métaphase, bien des mécanismes demeurent obscurs. L'entrée des cellules en phase M est sous la commande du facteur de promotion de la phase M ou MPF (*M Phase-Promoting Factor*). Identifié par Masui et Markert (1971), le MPF est une protéine kinase hétérodimère composée de deux sous-unités, l'une catalytique et stable, la $P34^{cdc2}$, et l'autre régulatrice synthétisée *de novo* à chaque cycle cellulaire, la cycline B. Quand la cycline B s'associe à la $P34^{cdc2}$, cette dernière est phosphorylée. La déphosphorylation d'une autre région de la $P34^{cdc2}$ par une phosphatase, produit du gène cdc^{25}, est cependant nécessaire pour compléter l'activation du MPF (fig. 2). Une fois activé, le MPF phosphoryle plusieurs protéines, dont les lamines, l'histone H1, la vimentine, soit des protéines qui provoquent la rupture de la membrane nucléaire, la condensation des chromosomes et la formation du fuseau mitotique. Même si tous les facteurs de l'entrée en phase M ne sont pas bien connus, on sait que la réplication complète de l'ADN est un préalable à cette entrée (revue par Nurse, 1990; Norbury et Nurse, 1992; Millar et Russell, 1992; Boulikas, 1995).

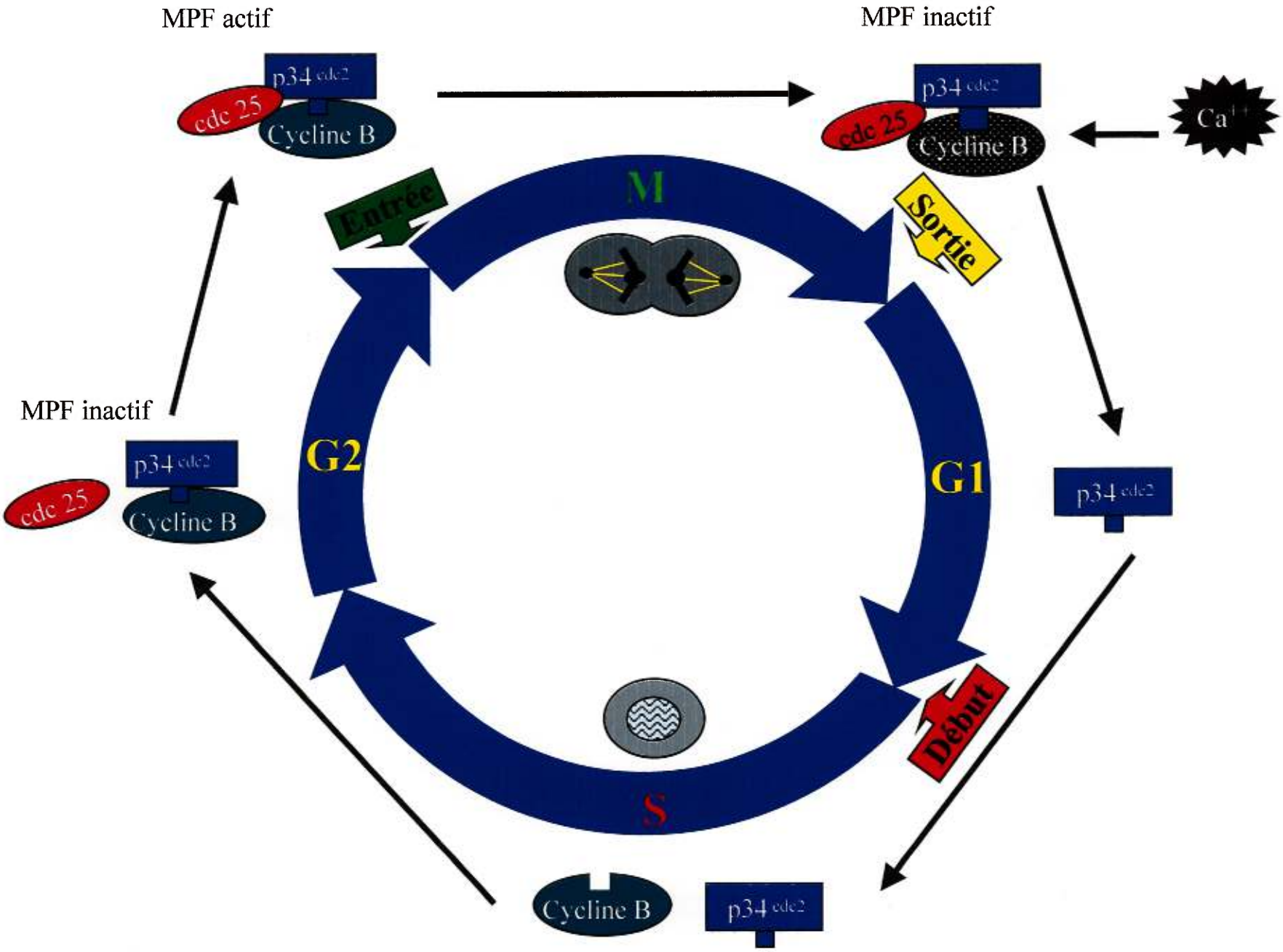
MPF actif
MPF inactif
p34 cdc2
cdc 25
Cycline B
Ca++
M
Entrée
Sortie
G1
S
G2
Début
MPF inactif
p34 cdc2
cdc 25
Cycline B
p34 cdc2
Cycline B
p34 cdc2

Figure 2.

Schéma de la régulation du MPF pendant le cycle cellulaire (d'après Norbury et Nurse, 1992). Pendant la phase S, la cycline B (portion régulatrice) est synthétisée et se lie à la P34cdc2 (portion active) pour former la MPF inactive. C'est la déphosphorylation de la P34cdc2 par la cdc25, qui est responsable de l'activation du MPF. Une fois activé, le MPF induit la phosphorylation de plusieurs protéines responsables de la condensation de la chromatine, de la dégradation de la membrane nucléaire, de la formation du fuseau mitotique et de division cellulaire. La dégradation de la cycline B est responsable de l'inactivation du MPF et donc de la sortie de la phase M.

La sortie de la phase M, qui comprend la séparation de la chromatine, la décondensation des chromosomes, le réassemblage de la membrane nucléaire et la cytocinèse, est déclenchée par la réduction des niveaux du MPF, elle-même dépendante de la dégradation de la cycline B (Murray, 1995; King et al., 1996b). La destruction de la cycline par protéolyse est calcium-dépendante (Lorca et al., 1991; Kono et al., 1996a; Whitaker, 1997). Une fois la métaphase terminée, les cellules peuvent poursuivre le cycle ou entrer dans un période de quiescence appelée G0, qui est probablement sous la commande de protéines kinases, dont une des plus importantes serait la $p27^{KIP1}$, et de facteurs de croissance (Sherr, 1996; Boulikas, 1995).

L'activation des mécanismes complexes qui permettent l'entrée des cellules en phase S, semble principalement sous la commande des kinases cyclines-dépendantes (CDK), dont la CDK2, la CDK4 et la CDK6. L'activation de ces cyclines se fait par liaison à la cycline E ou A (CDK2) ou encore à la D (autres CDK). Une fois activées, ces kinases phosphorylent certaines protéines, dont le produit du gène rétinoblastome, ce qui lève le blocage exercé par ces protéines sur les facteurs responsables de la réplication de l'ADN (Stillman, 1996; Sherr, 1996; Reed, 1997; Connell-Crowley et al., 1998).

3.3. La commande de la maturation méiotique des ovocytes

Au cours de la formation des gamètes féminins pendant l'embryogenèse, les cellules germinales se différencient en oogonies et se multiplient par mitose. Chez la plupart des mammifères, les oogonies se différencient pendant la vie fœtale en ovocytes, qui sont entourés d'une couche de cellules de la granulosa. Le passage d'oogonie à ovocyte marque le début de la méiose. Les substances responsables de

l'induction de la méiose (*méioses inducing substances; MIS)* n'ont pas encore été tout à fait identifiées. Cette maturation méiotique se poursuit jusqu'en prométaphase I aussi appelée dyctiotène ou stade de la vésicule germinative (VG), c'est-à-dire jusqu'à la réplication complète de l'ADN de chaque ovocyte (4n) et sa dispersion dans la membrane nucléaire. La prométaphase, qui peut s'étendre sur plusieurs années chez certaines espèces, prend fin au moment où les ovocytes réagissent au pic préovulatoire de LH. Ce dernier provoque la dégradation de la membrane nucléaire, la condensation de l'ADN, la formation du fuseau et l'expulsion du premier globule polaire. L'ovocyte demeure bloqué en métaphase II jusqu'à la fécondation (Wassarman et Albertini, 1994).

Le blocage en prométaphase peut être divisé en deux périodes : la première débute au moment où l'ovocyte se trouve dans le follicule primordial et se termine avec la fin de la croissance. L'arrêt de la méiose observé au cours de cette étape pourrait être imputable à la déficience de l'activité transcriptionnelle de l'ovocyte (Crozet et al., 1981; Motlik et Fulka, 1986; Crozet et al., 1986; Fair et al., 1995). La deuxième période commence avec la fin de la croissance de l'ovocyte et se termine quand survient le pic préovulatoire de LH ou lorsqu'on aspire l'ovocyte hors de son follicule. Il semble que les cellules folliculaires produisent des substances inhibitrices de la méiose (Eppig, 1994). La perte de contact entre les cellules de la granulosa (jonction ouverte) et l'ovocyte consécutive au pic préovulatoire de LH ou à l'aspiration de l'ovocyte empêcherait la transmission de la substance inhibitrice et provoquerait ainsi la reprise de la méiose (Wassarman et Albertini, 1994). Cependant, les mécanismes moléculaires en jeu ici ne sont pas encore élucidés. Des études ont montré que l'AMPc (Schultz et al., 1983; Bilodeau et al., 1993) , le Ca^{++} (Homa, 1995), les MAPK (*Mitogen-activated protein kinases* ou protéines kinases activées par des mitogènes) (Haccard et al., 1995; Chesnel et Eppig, 1995) et le MPF (Rime et al., 1992; Mitra et Schultz, 1996; de

Vantery et al., 1997), pourraient y jouer un rôle, dont l'importance et la nature sont encore mal comprises.

Malgré ces incertitudes, on croit que les étapes subséquentes seraient sous la commande du MPF, dont l'activité va croissante jusqu'en métaphase I pour ensuite diminuer puis à nouveau s'élever en métaphase II pour finalement retomber après la fécondation (fig.3) (Hashimoto et Kishimoto, 1988; Wu et al., 1997b; Sagata, 1997). Chez la plupart des mammifères, les ovocytes demeurent bloqués en métaphase II et pour reprendre leur développement, ils doivent être fécondés ou stimulés chimiquement (activation). Lors de la découverte du MPF, Masui et Markert (1971) ont proposé l'existence d'un facteur cytostatique (*Cytostatic Factor; CSF)* responsable du maintien des ovocytes au stade MII. Plus tard, Sagata et collaborateurs (1989) ont montré que le CSF, produit d'un proto-oncogène c-mos, était une phosphoprotéine. Cette dernière atteint une concentration maximale en MII pour disparaître après la fécondation (revue par Sagata, 1997). D'autres études ont montré que, outre la c-mos, ce CSF pourrait être constitué des kinases MAP et de la CDK2 (Gabrielli et al., 1993; Haccard et al., 1993). On ne sait pas encore comment cette protéine stabilise le MPF, mais ce pourrait être par inhibition de la régulation négative du MPF (Sagata, 1997).

3.4. L'activation des ovocytes

Lors de la fécondation, le spermatozoïde introduit son matériel génétique, mais il est également responsable de l'activation de l'ovocyte, un préalable au développement embryonnaire.

Activité MPF

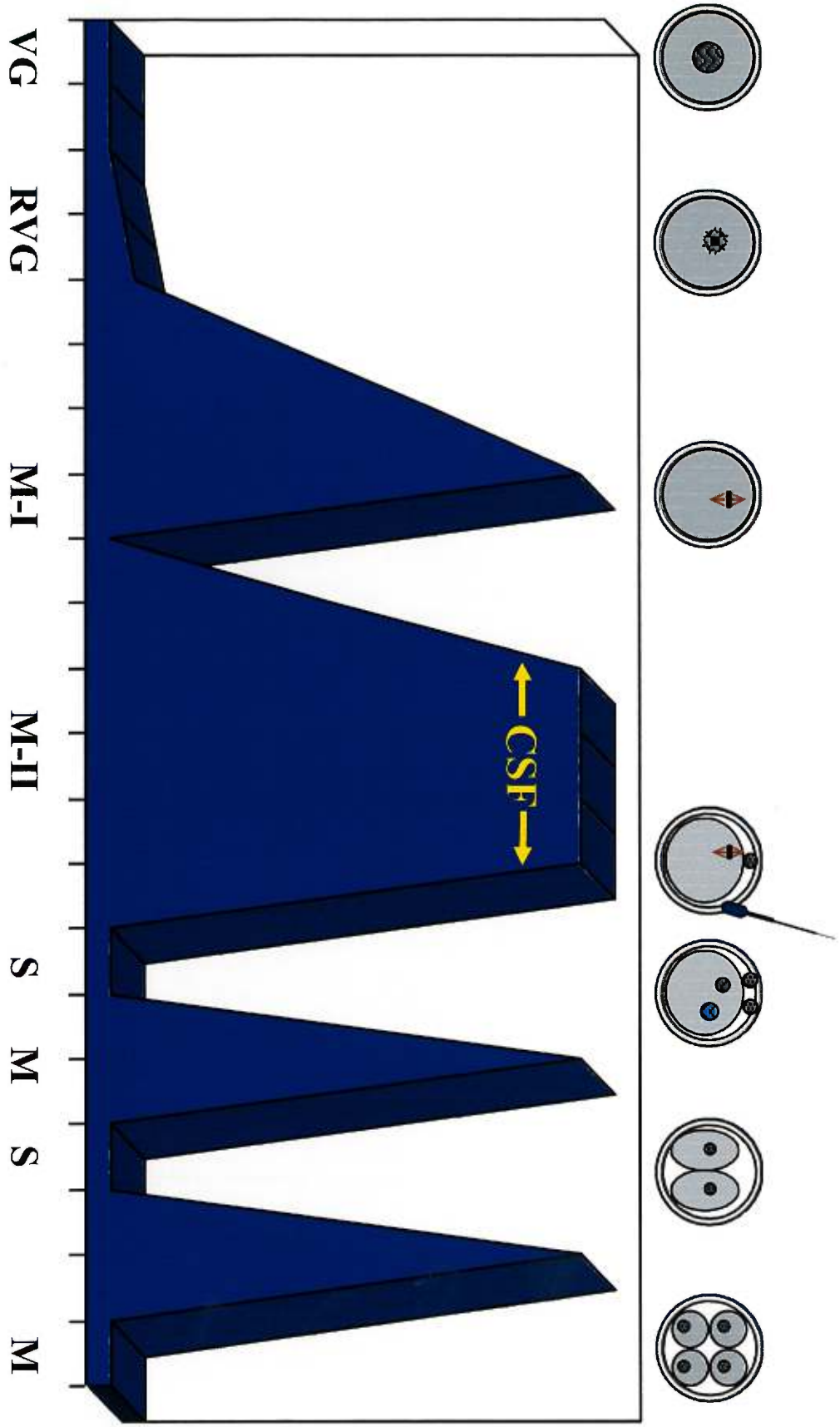

Figure 3.

Activité du MPF pendant la méiose et le début du développement embryonnaire (d'après Sagata, 1997). L'activité du MPF commence à augmenter au moment de la rupture de la vésicule germinale pour atteindre un niveau maximal en métaphase I. Il y a chute temporaire de l'activité lors de l'expulsion du premier globule polaire puis l'activité reprend rapidement et demeure élevée pendant le blocage de la méiose en métaphase II. Cette stabilisation du MPF est due à l'action du CSF (*cytostatic factor*). Les oscillations du calcium induites par le spermatozoïde à la suite de la fécondation ou par des agents chimiques entraînent l'inactivation du MPF, ce qui permet à l'ovocyte de compléter la maturation méiotique et d'éliminer le deuxième globule polaire. Le MPF est de nouveau activé à chaque cycle cellulaire pour permettre l'entrée en mitose.

La première réponse de l'ovocyte après la fécondation c'est la fluctuation du niveau de calcium (Jaffe, 1983), responsable de la dégradation de la cycline nécessaire pour la réduction du MPF (Lorca et al., 1993). Chez les mammifères, plusieurs études ont montré que le spermatozoïde induit une série d'oscillations du Ca^{++} qui perdurent par plusieurs minutes ou même des heures (Kline et Kline, 1992; Sun et al., 1992; Fissore et Robl, 1993; Shiina et al., 1993). Ces oscillations semblent être cruciales pour l'activation des ovocytes et la progression du développement embryonnaire.

Plusieurs méthodes d'activation artificielle des ovocytes ont déjà été testées chez différentes espèces (Nagai, 1987; Kubiak, 1989; Ware et al., 1989). En général, les résultats sont satisfaisants uniquement lorsque les ovocytes sont maintenus longtemps en culture avant d'être stimulés. Cela pourrait probablement s'expliquer par l'inefficacité de ces méthodes à induire les oscillations de Ca^{++}. En effet, les études de Ozil (1990), Vitullo et Ozil (1992) et de Collas et collaborateurs (1993; 1995) ont montré chez différentes espèces que l'induction de plusieurs oscillations du Ca^{++} est nécessaire pour provoquer la réduction du MPF et donc l'activation des ovocytes. Ceci confirme que les fluctuations du Ca^{++} produites par les spermatozoïdes sont nécessaires pour déclencher le développement embryonnaire chez les mammifères. Si les ovocytes *vieillis* sont moins exigeants à cet égard, c'est peut-être que la synthèse de la protéine kinase, produit du gène Mos et un des composants du facteur CSF, est moins importante (Wu et al., 1997a).

Il y a toutefois un revers à la médaille : en effet, s'ils sont plus faciles à activer (Heyman et al., 1994; Stice et al., 1994; Zakhartchenko et al., 1995), les ovocytes plus *vieillis* présentent toutefois un potentiel de développement amoindri (Chian et al., 1992; Kono et al., 1994). La mise au point de méthodes efficaces d'activation des jeunes ovocytes aux fins

de transfert nucléaire est donc souhaitable. La solution pourrait bien venir des études menées chez les amphibiens (Tompkins, 1978; Reinschmidt et al., 1979) et les poissons (Streisinger et al., 1981; Chourrout, 1982). En effet, des chercheurs ont démontré que des traitements d'irradiation inactivent complètement la chromatine des spermatozoïdes sans affecter leur potentiel de fécondation et d'activation des ovocytes. La fécondation des ovocytes par des spermatozoïdes irradiés est utilisée pour la manipulation de la ploïdie et la production de faux hybrides par gynogenèse chez ces espèces. De toutes les méthodes de neutralisation ou d'élimination de la chromatine des spermatozoïdes, l'irradiation aux ultraviolets est la plus utilisée parce qu'elle cause moins de fragmentation du ADN (Chourrout, 1984; Chourrout, 1986). Chez les mammifères, on a déjà employé l'irradiation aux ultraviolets pour induire l'énucléation des ovocytes hôtes (Tsunoda et al., 1988; Yang et al., 1990; Bradshaw et al., 1995), mais pas pour l'inactivation de la chromatine des spermatozoïdes. Si elle est efficace, cette technique pourrait s'avérer précieuse pour la manipulation du cycle cellulaires des ovocytes hôtes.

3.5. Le cycle cellulaire et le transfert nucléaire

Nos connaissances de base sur le rôle joué par le cycle cellulaire lors de la reconstitution d'embryons par transfert nucléaire proviennent des études de Johnson et Rao (1970) et Rao et Johnson (1970). Ces chercheurs ont montré que la fusion de cellules somatiques à différentes phases du cycle cellulaire donne lieu à différentes interactions. Plus capital encore, ils ont constaté que les cellules en métaphase induisaient la condensation prématurée de la chromatine (CPC) des cellules quelle que soit la phase du cycle cellulaire (G1, S et G2). Des études subséquentes ont montré que ce phénomène était imputable à l'activité du MPF présent dans les cellules en métaphase (revue par Rao, 1990). Il

faut bien sûr mentionner les études de Blow et Laskey (1988) et Leno et collaborateurs (1992), qui ont permis de démontrer que le cycle cellulaire pourrait jouer un rôle de premier plan lors de la reconstitution des embryons par transfert nucléaire. Ces études ont mis en évidence l'induction de la perméabilisation de la membrane nucléaire par le MPF ou d'autres substances, qui permet aux cellules de passer à nouveau par un cycle de réplication de l'ADN. L'intégrité de cette membrane serait donc nécessaire à la réplication normale de la chromatine pendant le cycle cellulaire. Dans la foulée de ces résultats, d'autres chercheurs ont tenté d'identifier les phases cellulaires idéales pour la reconstitution des embryons particulièrement au regard du potentiel de développement (Smith et al., 1988; Smith et al., 1990; Smith et Wilmut, 1994; Collas et al., 1992b; Cheong et al., 1993; Cheong et al., 1994; Campbell et al., 1994; Otaegui et al., 1994; Tsunoda et Kato, 1997) et des aspects morphologiques et fonctionnels de la chromatine après la reconstitution (Czolowska et al., 1984; Szöllösi et al., 1988; Collas et Robl, 1991; Collas et al., 1992a; Czolowska et al., 1992; Barnes et al., 1993; Campbell et al., 1993; Fulka et al., 1993; Cheong et al., 1994; Smith et al., 1996). On a pu en tirer deux conclusions : 1) les ovocytes hôtes en métaphase II peuvent endommager la chromatine et provoquer ainsi des problèmes de ploïdie sauf si les noyaux sont au stade G1 avant le début de la réplication de la chromatine et 2) si les ovocytes hôtes se trouvent en interphase, on peut effectuer le transfert sans égard à la phase du cycle cellulaire du noyau (revue par Campbell et al., 1996a; Fulka, Jr. et al., 1996). On le voit, l'ovocyte hôte préactivé était considéré comme un receveur universel. Cela demeure vrai chez les espèces où l'activation du génome embryonnaire se produit après quelques cycles cellulaires et dans les cas où on utilise des noyaux d'embryons en préimplantation. Cependant, chez la souris, où, comme on l'a vu, l'activation du génome embryonnaire survient pendant le premier cycle cellulaire et dans le cas, chez les autres espèces, où les noyaux donneurs proviennent de cellules

plus différenciées, il semblerait nécessaire d'avoir recours à des ovocytes hôtes en métaphase pour permettre une meilleure reprogrammation de la chromatine. Malgré les indications dont on dispose sur le rôle du cycle cellulaire sur la chromatine et le développement des embryons reconstitués, on saisit encore mal la nature de son action sur le remodelage des noyaux transplantés.

4. Le remodelage nucléaire chez les embryons reconstitués

Il semble bien que l'acquisition de la capacité de développement par les noyaux à transférer passe par une réorganisation morphologique et la reprogrammation des fonctions moléculaires. Les premières études réalisées chez les amphibiens ont montré que les noyaux transplantés subissent une décondensation et acquièrent une taille similaire à celle des pronoyaux présents dans les ovocytes fécondés (Subtelny et Bradt, 1963). Ce phénomène serait provoqué par l'importation de protéines cytoplasmiques dans les noyaux (Merriam, 1969). Par ailleurs, d'autres chercheurs ont mis en évidence des échanges de protéines entre les noyaux transplantés et les cytoplasmes hôtes pendant la période de croissance nucléaire (DiBerardino et Hoffner, 1975; Gurdon et al., 1976; Hoffner et DiBerardino, 1977).

Comme chez les amphibiens, on observe d'abord une forte augmentation du volume nucléaire chez les quelques espèces de mammifère étudiées jusqu'à présent (Czolowska et al., 1984; Collas et Robl, 1991; Prather et al., 1990). Des études subséquentes sur la reprogrammation nucléaire ont mis en évidence des modifications de la réplication de l'ADN (Campbell et al., 1993; Barnes et al., 1993; Pinto-Correia et al., 1995), de l'activité transcriptionelle (Dyban et al., 1988; Kanka et al., 1991; Pinto-Correia et al., 1995; Kanka et al., 1996; Smith

et al., 1996; King et al., 1996a), de la synthèse des protéines (Howlett et al., 1987; Latham et al., 1991), et de l'expression de certains antigènes modifiés au cours du développement embryonnaire (Prather et al., 1991; Prather et Rickords, 1992; van Stekelenburg-Hamers et al., 1994; Pinto-Correia et al., 1995). De manière générale, ces études indiquent qu'après la transplantation, les noyaux sont ainsi remodelés dans le cytoplasme hôte et que la valeur de ces paramètres est similaire à celle qu'on observe chez les embryons avant la première division. Par contre, l'examen des patrons de synthèse protéinique après transfert nucléaire chez la souris montre que les noyaux provenant d'embryons de 8 blastomères ne peuvent être ramenés au stade 2 blastomères en ce qui a trait à la synthèse protéinique (Latham et al., 1994). En outre, les embryons bovins reconstitués présentent, au stade 1, 2 ou 4 cellules, des patrons de transcription de l'ARN hétérogène différents de ceux des embryons issus de la fécondation *in vitro* (Lavoir et al., 1997a). Il est indiscutable, malgré ces controverses, que les ovocytes hôtes sont capables de modifier la morphologie et les fonctions des noyaux transplantés. Reste à déterminer les facteurs responsables de ces modifications et le rôle de celles-ci dans la reprogrammation.

Certaines études suggèrent que le stade cellulaire des ovocytes hôtes jouerait un rôle dans le remodelage des noyaux transférés. En effet, la décondensation et la croissance nucléaire se déroulent mieux quand les noyaux sont transférés dans des ovocytes hôtes en métaphase plutôt qu'interphase (Czolowska et al., 1984; Collas et Robl, 1991). De plus, les noyaux provenant d'embryons de 16 blastomères, qui ne se lient pas à l'anticorps anti-lamines A/C (J9), acquièrent l'antigène une fois transplantés dans des ovocytes en métaphase. Cependant, lorsque les ovocytes hôtes sont en interphase, les anticorps marqués sont faiblement détectés (Prather et al., 1991). Finalement, l'activité transcriptionnelle des noyaux transplantés telle que mesurée par l'incorporation de ^{3}H-

uridine s'est interrompue beaucoup plus rapidement lorsque l'ovocyte hôte se trouvait en métaphase que lorsqu'il était en interphase (Smith et al., 1996). Quoi qu'il en soit, le rôle du cycle cellulaire des ovocytes hôtes sur le remodelage de la chromatine du noyau donneur devra être démontré plus clairement.

5. Les modifications subies par la chromatine pendant le développement et ses conséquences potentielles sur le déroulement du transfert nucléaire

Les cellules déjà en voie de différenciation (Smith et Wilmut, 1989; Campbell et al., 1996b) ou même déjà spécialisées (tissus adultes) (Wilmut et al., 1997; Wakayama et al., 1998; Kato et al., 1998), maintiennent donc leur totipotence puisqu'elles peuvent servir à produire des clones. Cependant, on devra éclaicir l'impact des mécanismes qui commandent la différenciation et l'activité de la chromatine sur le remodelage nucléaire lors de la reconstitution des embryons.

Parmi les modifications structurelles qui provoquent un remaniement de l'activité de la chromatine au cours du développement et de la différenciation cellulaire, on retrouve la méthylation d'une base, la cytosine (Kass et al., 1997) ainsi que l'altération de certaines des protéines qui forment les nucléosomes, soit l'acétylation des histones centrales (Wade et al., 1997) et le remplacement des histones de liaison (Clarke et al., 1998). Quelles sont les conséquences de ces modifications sur le potentiel nucléaire de reprise du développement et par quels mécanismes l'ovocyte hôte tire-t-il partie de ces modifications? Ce genre de question pourrait nous amener à expliquer comment les cellules différenciées recouvrent leur totipotence.

La méthylation de l'ADN est probablement une des plus importantes modifications épigénétiques, car elle a des effets spectaculaires sur l'activité de la chromatine pendant la différenciation cellulaire (Kass et al., 1997). Elle commande ainsi l'expression de l'empreinte génomique, c'est-à-dire de l'expression différentielle des allèles selon leur origine maternelle ou paternelle (revue par Latham et al., 1995; Reik et Walter, 1998). La méthylation contribue aussi à l'inactivation d'un des chromosomes X chez les femelles (Goto et Monk, 1998) de même qu'à l'inhibition de la transcription de plusieurs gènes devenus inutiles après un certain stade de différenciation cellulaire (Bird, 1995).

L'importance de l'empreinte génomique dans le développement embryonnaire chez les mammifères a été mise en évidence par des études sur le transfert nucléaire (McGrath et Solter, 1984a; Surani et al., 1984; Surani et al., 1986), qui ont montré que les embryons reconstitués avec deux pronoyaux mâles (androgéniques) ou femelles (gynogéniques) n'était pas viables. Plus récemment, lors d'études sur des embryons gynogéniques effectuées par la transplantation de la chromatine d'ovocytes immatures dans des ovocytes matures, il a été démontré que la modification de certaines empreintes géniques au cours de la croissance des ovocytes interfère avec le temps de survie des embryons reconstitués (Kono et al., 1996b; Obata et al., 1998). D'autres ont montré que la fusion entre cellules germinales (CG) embryonnaires et cellules somatiques du thymus se traduisait par une déméthylation de plusieurs gènes avec ou sans empreinte. Les CG seraient donc capables de reprogrammer les patrons de méthylation des cellules somatiques (Tada et al., 1997). Il reste cependant à déterminer comment les patrons de méthylation de l'ADN sont modifiés lors du transfert des noyaux dans des ovocytes hôtes.

L'acétylation des régions amino-terminales des histones centrales des nucléosomes est une autre modification notable, qui sans toucher directement la chromatine affecte grandement l'activité transcriptionnelle. Contrairement à la méthylation de la cytosine, l'acétylation des histones centrales active la transcription (Turner, 1991; Wade et al., 1997). Selon des résultats plus récents, la méthylation et l'acétylation agissent conjointement sur la commande de l'activité transcriptionnelle de la chromatine (Nan et al., 1998; Jones et al., 1998). Adenot et collaborateurs (1997) ont suivi chez la souris à l'aide d'un anticorps qui reconnaît spécifiquement les formes hyperacétylées de l'histone centrale H4, les changements subis par cette protéine au début du développement embryonnaire. Ils ont démontré que la chromatine des ovocytes en métaphase et des spermatozoïdes n'est pas marquée par l'anticorps. L'histone H4 hyperacétylée peut déjà être identifiée d'abord dans le pronoyau mâle puis dans le pronoyau femelle durant le premier cycle cellulaire. Ces résultats suggèrent qu'il y aurait un lien entre l'acétylation des histones et le début de l'activité transcriptionnelle puisque chez la souris des transcrits embryonnaires sont détectés au premier cycle cellulaire et en plus grande quantité dans le pronoyau mâle (Aoki et al., 1997). On ne recense aucun article sur les effets de l'acétylation des histones lors du transfert nucléaire.

Tout comme les histones centrales, les histones de liaison ou histone H1 ont elles aussi un impact sur l'activité de la chromatine (revue par Wolffe, 1996; Clarke et al., 1998). Ces protéines font partie de l'organisation structurale de la chromatine et elles interagissent avec l'ADN situé entre les nucléosomes adjacents (ADN de liaison; fig. 4).

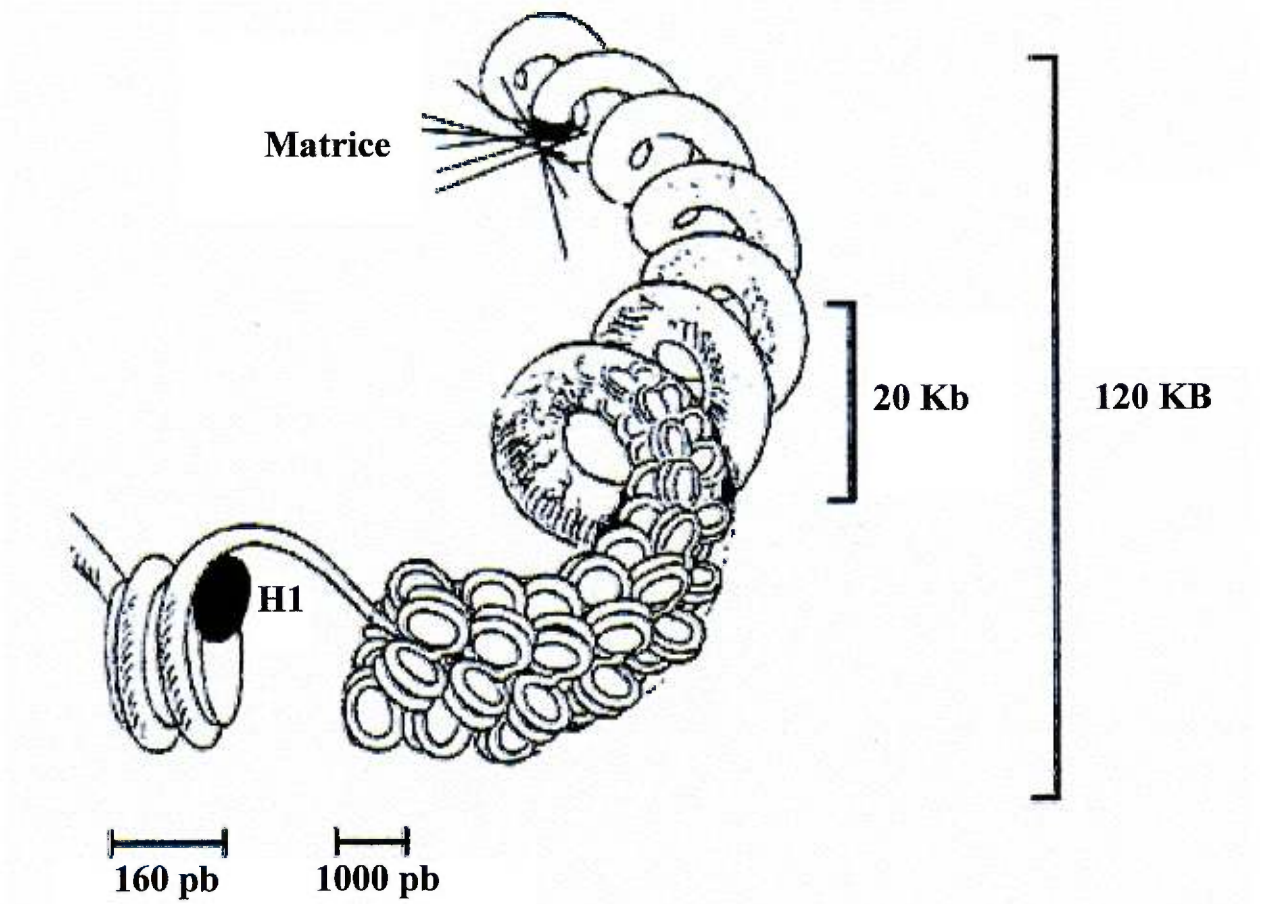

Figure 4.

Représentation schématique de la localisation de l'histone somatique H1 dans la chromatine (d'après Wolffe, 1996). L'histone H1 se situe entre le nucleosome et elle est en contacte avec l'ADN de liaison.

Jusqu'à maintenant, on a identifié sept différents sous-types chez les mammifères : H1a, H1b, H1c, H1d, H1e, $H1^0$ et H1t. Les cinq premiers (H1a-e) sont dits sous-types somatiques et représentent quasiment la totalité des H1 présents dans les cellules somatiques en réplication. Le sous-type $H1^0$ dit de remplacement s'accumulent dans les cellules en arrêt de prolifération et en début de différenciation. Enfin, le H1t est présent uniquement dans les spermatocytes (Wolffe et al., 1997; Clarke et al., 1998).

Au cours du développement embryonnaire, les histones somatiques H1 de liaison se substituent aux histones embryonnaires. Ces derniers bien caractérisés chez la grenouille (Cho et Wolffe, 1994) et l'oursin de mer (Mandl et al., 1997) le sont moins chez les mammifères. Clarke et collaborateurs (1997) croient que, chez la souris, la $H1^o$ serait le sous-type embryonnaire. Un important aspect de cette substitution est que l'assemblage des formes somatiques dans la chromatine coïncide avec la principale période d'activation du génome embryonnaire tel que démontré chez les amphibiens (Ohsumi et Katagiri, 1991; Dimitrov et al., 1993) et différentes espèces de mammifères (Clarke et al., 1992; Smith et al., 1995). Il y aurait donc un lien entre le remplacement des histones H1 et cette importante étape du développement embryonnaire. Cependant, les études ont révélé aussi bien une inhibition qu'une activation de l'expression de certains gènes par l'histone H1 (revue par Wolffe et al., 1997). Pourtant, chez la grenouille (*Xenopus leaves)*, la modification expérimentale du moment du remplacement des types embryonnaires B4 par le type somatique H1 affecte le moment de l'expression des gènes responsables de la formation du mésoderme embryonnaire. Cela indique bien l'importance de la H1 somatique dans la régulation de l'activité du génome embryonnaire (Steinbach et al., 1997).

La régulation fonctionnelle de la chromatine au cours de l'activation du génome embryonnaire semble reposer, du moins partiellement, sur l'assemblage des histones H1 somatiques, mais on ne sait pas comment ces dernières modifient l'activité de la chromatine. Lors de l'activation du génome embryonnaire il y a répression marquée de l'activité de la chromatine qui se traduit par une réduction de l'action des différents promoteurs (revue par Nothias et al., 1995). Cette répression est probablement occasionnée par des modifications de la structure de la chromatine, dont l'acétylation des histones centrales et le remplacement des histones H1 (Turner, 1991; Ura et al., 1994; Kingston et al., 1996; Wiekowski et al., 1997). Certaines observations semblent indiquer que les histones H1 seraient en mesure de réduire la mobilité des nucléosomes, c'est-à-dire de diminuer l'accès à la chromatine et, donc, de modifier l'activité transcriptionnelle (Pennings et al., 1994; Ura et al., 1996; Kingston et al., 1996; Ura et al., 1997).

Dimitrov et Wolffe (1996) ont démontré que, chez la grenouille, les noyaux d'érythrocytes subissent plusieurs altérations moléculaires, dont le désassemblage des histones H1 somatiques et l'assemblage des histones de liaison embryonnaires B4 lorsqu'ils sont transférés dans des extraits d'ovocytes. Cette substitution a un impact notable sur la transcription des gènes de l'ARNm 5S. Ces résultats suggèrent que les modifications subies par les histones H1 pourraient constituer un élément crucial de la reprogrammation fonctionnelle de la chromatine des noyaux transplantés. Quand nous saurons si l'histone H1 somatique est remplacé lors du transfert nucléaire et quels facteurs président à ce remplacement, nous comprendrons mieux le phénomène du retour à la totipotence des noyaux transplantés.

Les études de Philpott et collaborateurs (1991) et Philpott et Leno (1992) avec des extraits d'ovocytes de *Xenopus* ont mis en lumière les

facteurs en jeu dans le remodelage de la chromatine des spermatozoïdes nécessaires à la formation du pronoyau mâle. Ils ont démontré que l'élimination de certaines protéines spermatiques et l'assemblage des histones centrales étaient sous la commande des nucléoplasmines. Ces protéines sont également responsables du désassemblage de l'histone somatique H1 de la chromatine dans les noyaux de cellules somatiques transférés dans des extraits d'ovocytes de *Xenopus* (Dimitrov et Wolffe, 1996). Autre aspect intéressant de ces nucléoplasmines, leur activité est probablement sous la commande des kinases en jeu dans la régulation du cycle cellulaire. En effet, Sealy et collaborateurs (1986) ont montré que les nucléoplasmines étaient phosphorylées lors de la maturation méiotique des ovocytes et que leur potentiel de remodelage de la chromatine des spermatozoïdes dépendait de cette phosphorylation (Leno et al., 1996). Ces importantes observations moléculaires nous aideront à comprendre comment les ovocytes hôtes agissent sur la chromatine des noyaux transplantés. Bien qu'aucun mécanisme semblable n'ait encore été décrit chez les mammifères, McLay et Clarke (1997) ont montré que la capacité de remplacer des protamines par des histones dans la chromatine des spermatozoïdes est acquise au cours de la maturation méiotique des ovocytes. En outre, le stade cellulaire des ovocytes a un impact sur le remodelage de la chromatine des spermatozoïdes (Borsuk et Tarkowski, 1989) et sur celui des noyaux somatiques (Czolowska et al., 1984; Szöllösi et al., 1988). La reprogrammation de la chromatine chez les mammifères pourrait bien être tributaire de mécanismes soumis aux mêmes régulations que chez les amphibiens.

III. INTÉRET DU TRAVAIL

En raison de son grand intérêt pour la recherche et l'industrie, le transfert nucléaire attire l'attention de plusieurs chercheurs à travers le monde. La percée la plus spectaculaire associée à cette technique est sans aucun doute le clonage d'un mammifère adulte (Wilmut et al., 1997). Bien que l'efficacité du transfert nucléaire pour produire des animaux identiques à partir de noyaux provenant d'embryons en pré-implantation ait été établie dans les années 80 (Willadsen, 1986), ce sont les récents succès obtenus avec des cellules somatiques qui ont ravivé l'intérêt de l'industrie, particulièrement le secteur bio-pharmaceutique, envers cette technique. L'importance de cette technologie a été soulignée par de récentes études, qui ont démontré que des cellules somatiques en culture ayant reçu un gène exogène étaient capables de produire des individus transgéniques par transfert nucléaire (Schnieke et al., 1997; Cibelli et al., 1998). Dans l'immédiat, l'application la plus intéressante de cette observation serait l'utilisation du transfert nucléaire pour la production d'animaux à partir de cellules transfectées en vue de produire des protéines pharmaceutiques et pour la modification génétique d'animaux en vue de la transplantation de leurs organes à l'homme (Stice et al., 1998). Par ailleurs, le transfert nucléaire pourrait conduire à l'augmentation du rythme de l'amélioration génétique des troupeaux grâce au clonage des animaux adultes au potentiel de production connu. À plus long terme et une fois approfondies nos connaissances du génome de chaque espèce, cette technologie pourrait servir à produire des animaux pourvus de certains caractères précis reliés, par exemple, à la résistance aux maladies, à la qualité de viande, aux teneurs en gras et en protéine du lait, au taux de croissance, à la reproduction, à l'indice de conversion alimentaire et même au comportement.

Au-delà de son potentiel commercial, le transfert nucléaire occupe une place de choix en recherche fondamentale : c'est grâce au

remplacement nucléaire qu'a été démontrée l'importance des empreintes géniques dans le développement embryonnaire (Surani et al., 1984; McGrath et Solter, 1984a). On pourra aussi élucider davantage les mécanismes de commande de la ségrégation mitochondriale chez les mammifères (Meirelles et Smith, 1997). Enfin, cette technique favorisera sans doute une meilleure compréhension des modifications épigénétiques subies par la chromatine au cours de la différenciation cellulaire et de leur impact sur la totipotence nucléaire.

Jusqu'à maintenant, cependant, les résultats obtenus ne sont pas à la hauteur des attentes. Il faudra donc identifier les mécanismes qui permettront d'arriver à des résultats uniformes et satisfaisants. Au moins une partie de la solution pourrait résider dans une meilleure connaissance des interactions nucléocytoplasmiques en cause lors de la reconstitution des embryons. Les études de Smith et collaborateurs (1988; 1990) chez la souris ont pour la première fois mis en évidence l'importance de la commande du cycle cellulaire sur le développement des embryons issus d'un transfert nucléaire. Par la suite, plusieurs autres études ont confirmé ces résultats chez différentes espèces (Collas et Robl, 1991; Collas et al., 1992b; Cheong et al., 1993; Barnes et al., 1993; Campbell et al., 1994).

Comme la majorité des blastomères se trouvent en phase S (phase de synthèse de l'ADN) du cycle cellulaire pendant le développement embryonnaire qui précède l'implantation (Barnes et al., 1993; Campbell et al., 1994) l'utilisation d'ovocytes hôtes activés, c'est-à-dire chez qui on a induit la sortie de l'état dit d'arrêt métaphasique de la méiose, pourrait être un facteur déterminant de succès lors du transfert de noyaux de blastomères (Campbell et al., 1996a). Ainsi, chez certaines espèces, la mise au point de procédures efficaces pour l'activation des ovocytes hôtes est devenue cruciale pour le clonage à partir de cellules embryonnaires.

Cependant, des études plus récentes chez la souris suggèrent que le transfert de noyaux de cellules embryonnaires ou adultes ayant séjourné pendant un certain temps dans des ovocytes hôtes en métaphase II avant l'activation de ces derniers, pourrait expliquer les succès obtenus chez cette espèce (Kwon et Kono, 1996; Wakayama et al., 1998). Il est donc fondamental de bien établir le rôle des différents stades du cycle cellulaire sur le remodelage des noyaux transplantés. C'est la réponse à ce genre de question qui nous conduira à la mise au point de méthodes de clonage plus efficaces tant au regard de la recherche fondamentale que des applications commerciales.

IV. HYPOTHÈSE ET OBJECTIFS DU TRAVAIL

1. Hypothèse

Le stade du cycle cellulaire des ovocytes hôtes et des cellules donneuses joue un rôle important dans le remodelage de la chromatine transplantée, qui se déroule pendant les interactions nucléocytoplasmiques qui surviennent après un transfert nucléaire.

2. Objectifs

L'objectif général de ce travail était de déterminer l'importance du cycle cellulaire lors de la reconstitution d'embryons par transfert nucléaire. Quatre projets ont été conçus à cette fin. Les deux premiers de nature plus appliquée portaient sur la mise au point de méthodes efficaces d'activation des ovocytes hôtes et de contrôle de leur potentiel dans une perspective de clonage. Les autres orientés vers la biologie du développement avaient trait à l'étude du remodelage de la chromatine dans les embryons produits par transfert nucléaire.

Les objectifs spécifiques à chaque projet sont les suivants :

A. Mettre au point des méthodes efficaces d'induction de l'activation des ovocytes bovins et déterminer si ces derniers permettent d'améliorer l'efficacité de la technique de clonage d'embryons (article 1).

B. Déterminer si les spermatozoïdes irradiés aux ultraviolets peuvent être utilisés pour induire l'activation des jeunes ovocytes non *vieillis* et vérifier si ces derniers permettent d'obtenir un meilleur taux de développement des embryons reconstitués par transfert nucléaire (article 2).

C. Vérifier, chez les embryons bovins reconstitués par transfert nucléaire, si l'histone somatique H1 est remodelée après la fusion du blastomère avec l'ovocyte hôte à différents moments de l'activation (article 3).

D. Déterminer, chez les embryons de souris produits par transfert nucléaire, l'importance du stade du cycle cellulaire des ovocytes hôtes et des noyaux transplantés dans le remodelage de l'histone somatique H1 (article 4).

V. MATÉRIEL, METHODES ET RÉSULTATS

Article 1

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Telophase Enucleation: An Improved Method to Prepare Recipient Cytoplasts for Use in Bovine Nuclear Transfer

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ABSTRACT The enucleation of oocytes to be used as host cytoplasts for embryo reconstruction by nuclear transfer is an important limiting step when cloning mammals. We propose an enucleation technique based on the removal of chromatin after oocyte activation, at the telophase stage, by aspirating the second polar body and surrounding cytoplasm. In a preliminary experiment to determine an optimal activation protocol, oocytes were matured for 26 and 30 hr and exposed for 5 min to 7% ethanol and/or for 3 hr at either 25 or 4°C. Relative to most activation treatments tested, oocytes matured for 30 hr and exposed to ethanol alone showed highest activation rates, as determined by low levels of H1 kinase activity within 90 min from exposure and high pronuclear formation (82%) after 12 hr of culture. No synergistic effect on activation rates was observed when oocytes also were exposed to reduced temperature after ethanol treatment. Microsurgical removal of the telophase-stage chromatin in a small volume of cytoplasm adjacent to the second polar body was significantly more effective in enucleating than aspiration of a larger cytoplasm volume surrounding the first polar body of metaphase-arrested oocytes (98% versus 59%; $P < 0.01$). Moreover, compared with a nuclear transfer protocol based on enucleation of metaphase-arrested oocytes followed by aging and cooling, more (38% versus 16%; $P < 0.001$) and better-quality blastocytes (126 versus 84 nuclei per blastocyst; $P < 0.02$) were obtained from embryos reconstructed using the telophase procedure. Higher development potential of embryos reconstructed by the telophase procedure may be attributed to (1) the selection of oocytes that activate and respond by extruding the second polar body, (2) avoiding the use of DNA dyes and ultraviolet irradiation, and (3) the limited removal of cytoplasm during enucleation. The ease with which telophase enucleation can be performed is likely to render this technique widely useful for research and practice on mammalian cloning. *Mol. Reprod. Dev. 49:29–36, 1998.* © 1998 Wiley-Liss, Inc.

Key Words: telophase enucleation; oocyte activation; histone H1 kinase; nuclear transfer; bovine

INTRODUCTION

Cell cycle–dependent nucleocytoplasmic interactions are known to influence profoundly the development potential of reconstructed oocytes produced by nuclear transfer in several mammalian species (Campbell et al., 1993; Collas and Robl, 1991; Smith et al., 1988; Tarkowski and Balakier, 1980). Although progress has been made to alter the cell cycle stage of blastomeres used as nuclear donors (Kato and Tsunoda, 1992; Otaegui et al., 1994; Samaké and Smith, 1995), reports using cattle embryos have shown limited ability to identify completely effective and nontoxic protocols for blastomere synchronization (Samaké and Smith, 1996, 1997). Another somewhat simpler way to reduce nucleocytoplasmic incompatibilities in nuclear transfer is to manipulate the cell cycle kinases present in the host cytoplasm at the time of reconstruction. Maturation-promoting factor (MPF) is the main cell cycle kinase activity responsible for nuclear envelope breakdown and chromatin condensation in mitotic and meiotic cells (Nurse, 1990). Since most morula-stage blastomeres are in interphase at any specific time of development, host cytoplasm free of MPF activity provides the benefit of the integrity of the nuclear envelope that protects the donor chromatin from harmful effects caused by cell cycle asynchrony (Barnes et al., 1993; Campbell et al., 1993).

The inactivation of MPF in metaphase-arrested oocytes occurs within a short period after penetration by the fertilizing spermatozoa or after parthenogenetic activation using artificial stimuli (Collas et al., 1993). In cattle, as in other mammals, parthenogenetic activation is dependent on the length of time metaphase oocytes remain arrested before exposure to the activation stimulus (Kubiak, 1989; Nagai, 1987; Ware et al., 1989). As oocytes age in the oviduct or in culture, the putative cytostatic factor (CSF) responsible for stabilizing MPF is degraded, leading to an increasingly prompt activation by exposure to environmental stimuli, such

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as ethanol, Ca^{2+}-ionophore, electric pulse, etc. Since temperature reduction also has been shown to induce activation of aged oocytes in some mammals (Ben-Yosef et al., 1995; Powell and Barnes, 1992; Stice et al., 1994), aging metaphase-enucleated oocytes followed by cooling for a short period immediately before embryo reconstruction by nuclear transfer appears beneficial (Ectors et al., 1995; Heyman et al., 1994; Stice et al., 1994; Zakhartchenko et al., 1995).

Nonetheless, development to the blastocyst stage in vitro and establishment and continuity of pregnancy from embryos originated by nuclear transfer remain poor (Kruip and den Daas, 1997). Several steps in the nuclear transfer procedure could be harmful to embryonic development. Although easier to activate, aged oocytes have significantly lower potential to support development after fertilization (Chian et al., 1992). Moreover, enucleation of metaphase-stage oocytes requires the use of DNA-specific vital stains together with ultraviolet (UV) irradiation to visualize the chromatin either before removal or for ascertaining removal after blind enucleation (Prather et al., 1987; Smith, 1992; Westhusin et al., 1992). Further, nearly one-third of the oocyte cytoplasmic volume is microsurgically removed to ensure high enucleation rates. Lastly, although oocytes enucleated at metaphase are exposed to activation, some oocytes may fail to do so, resulting in incompatible interactions after fusion to blastomeres in interphase. Therefore, the objective of this study was to establish a reliable oocyte activation protocol for nonaged oocytes to be used in obtaining recipient cytoplasts without the limitations described above. We hereby report on a simple enucleation method using telophase-stage activated oocytes that avoids DNA staining and removal of large quantities of cytoplasm. Moreover, development of reconstructed oocytes to the blastocyst stage is higher than with a standard nuclear transfer procedure, supporting the potential of this technique for cattle and other mammals.

MATERIALS AND METHODS

Oocyte Maturation, Fertilization, and Culture In Vitro

Ovaries were collected from a local abattoir, stored in saline at 30 to 35°C, and brought to the laboratory within 2 hr of slaughter. Follicles with diameters between 2 and 8 mm were punctured with a 19-gauge needle, and cumulus-oocyte complexes (COCs) with several layers of cumulus cells and homogeneous cytoplasm were washed in HEPES-buffered tissue culture medium (TCM-199; Gibco BRL, Burlington, Canada) supplemented with 10% (v/v) fetal calf serum (FCS; Gibco). Groups of 20 COCs were placed in 100 µl of bicarbonate-buffered TCM-199 supplemented with 10% FCS, 50 µg/ml LH (Ayerst, London, ON, Canada), 0.5 µg/ml follicle-stimulating hormone (Folltropin-V; Vetrepharm, St. Laurent, Quebec, Canada), 1 µg/ml estradiol-17β (Sigma, St. Louis, MO), 22 µg/ml pyruvate (Sigma), and 50 µg/ml gentamicin (Sigma). After 24 hr of maturation in vitro (IVM), oocytes were in vitro fertilized (IVF) using standard protocols. Briefly, COCs were placed in 50-µl drops of Tyrode's medium, supplemented with 0.6% bovine serum albumin (BSA; Fraction V, Sigma), lactate, pyruvate, gentamicin, and 10 µg/ml heparin (Parrish et al., 1986). Frozen-thawed spermatozoa were washed and centrifuged through a Percoll gradient and diluted at 10^6 live spermatozoa per milliliter. At 20 hr after insemination, COCs were denuded of cumulus cells by brief shaking, and the presumed fertilized zygotes were transferred to 50-µl drops of Menezo's B2 medium (MB2; Pharmascience, Paris, France) supplemented with 10% FCS in the presence of bovine oviductal epithelial cells (BOEC). All the cultures were performed in drops under equilibrated mineral oil at 39°C in a humidified atmosphere of 5% CO_2 in air.

Oocyte Activation Protocol

To establish an effective protocol for activating oocytes at both 26 and 30 hr of IVM, cumulus cells were removed from COCs by vigorous shaking in Dulbecco's phosphate-buffered saline (DPBS; Gibco) with 2 mg/ml hyaluronidase (Sigma), and denuded oocytes with a first polar body were randomly selected and treated in a 2 × 6 experiment with 7 replicates as follows: (1) Control nonmanipulated oocytes, (2) 3 hr of exposure to room temperature (RT; approximately 25°C), (3) 3 hr of exposure to 4°C (T-4), (4) 5 min in 7% ethanol (Eth), (5) ethanol with 3 hr of RT, and (6) ethanol with 3 hr of 4°C. After treatment, nontreated control and treated oocytes were cultured in TCM-199 for a further 12 hr before fixation. All oocytes were fixed in 10% formalin for 20 min, stained with a DNA-specific fluorochrome (5 µg/ml of Hoechst 33342; Sigma), mounted onto glass slides, and examined by epifluorescence. Oocytes with one or two pronuclei were considered activated.

Histone H1 Kinase Activity Assay

Protein kinase assays were performed according to methods previously described (Chesnel and Eppig, 1995). Groups of 10 oocytes were collected at 1.5 and 3 hr after activation in 10 µl of kinase buffer containing 60 mmol β-glycerophosphate, 30 mmol/L *p*-nitrophenylphosphate, 25 mmol/L 3-[*N*-morpholino]propanesulfonic acid (MOPS, pH 7.2), 15 mmol/L ethyleneglycoltetraacetic acid (EGTA), 15 mmol/L $MgCl_2$, 0.1 mmol/L sodium orthovanadate (Na_3VO_4), 1 mmol/L dithiotheitol (DTT), 1 mmol/L phenyl-methyl-sulfonyl fluoride (PMSF), and 1 mg/ml bovine serum albumine (BSA) and quickly frozen at −70°C. After thawing, lysates were centrifuged at 15,000 rpm for 10 min at 4°C before use. Kinase reactions were carried out for 1 hr at 30°C in a total volume of 25 µl in H1 kinase buffer supplemented with 100 µg/ml histone H1 (type III-S; Sigma), 1 µg/ml cAMP-dependent protein kinase A inhibitor (Sigma), and 40 µCi/ml [γ-^{32}P]ATP. Reactions were terminated by adding 25 µl of double-strength Laemmli sample buffer (Laemmli, 1970). Phosphorylation of substrate was analyzed by 15% sodium dodecyl

sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the gels were fixed for at least 1 hr (two washes) in 50% methanol–10% acetic acid, briefly washed in distilled water, dried on Whatman paper sheet at 60°C for 90 min, and exposed to x-ray film at −70°C for 24 hr. The evaluation of histone H1 phosphorylation was performed by densitometric analysis of the autoradiogram using a laser densitometer (Ultrascan XL, Pharmacia) with accompanying software (Gelscan XL software Package, Pharmacia). Five replicates per group, each comprising 10 oocytes, were evaluated, and the histone H1 activity was expressed as percentage in relation to the nonactivated control 26-hr IVM group from the same gel.

Chromatin and Spindle Staining

Oocytes were fixed in 10% formalin (Sigma) for 20 min, washed, and stored in PBS at 0.1% BSA and 0.5% Triton-100X at 4°C. Spindle-specific staining was obtained by incubating fixed oocytes in a blocking solution (PBS, 3% BSA, 0.1% Tween-20, 10% goat serum, and 10% horse serum) for 30 min at room temperature. These were then transferred to primary antibody raised against mouse α-tubulin diluted in blocking solution at 1:1500 and incubated in a humidified chamber overnight at 4°C. This step was followed by double 15-min washes in PBS and 0.1% Tween and incubation in fluorescence-conjugated goat antimouse IgG (diluted 1:100 in PBS and 0.1% Tween) for 1 hr at room temperature. After processing and washing, oocytes were mounted on slides in a drop of Mowiol (Aldrich, Milwaukee, WI) containing the DNA-specific dye bisbenzimide at 1 μg/ml (Hoechst 33342; Sigma). Oocyte chromatin and spindle morphology was examined by epifluorescence using a filter block at 380 nm excitation and 420 nm emission (UV-2A; Nikon, Tokyo).

Oocyte Reconstruction Protocols

Two oocyte reconstruction protocols were compared. In the standard protocol ("aged" group) (Heyman et al., 1994; Stice et al., 1994; Zakhartchenko et al., 1995), oocytes were denuded of cumulus cells at 24 hr of IVM, placed in micromanipulation PBS containing 7.5 μg/ml cytochalasin B (Sigma), and approximately 30% of the cytoplasm surrounding the first polar body was removed. After microsurgery, oocytes were placed in medium containing 5 μg/ml Hoechst 33342 for 15 min and exposed briefly to UV irradiation to verify the absence of chromatin and thus complete enucleation. Enucleated oocytes were returned to IVM drops for another 18 hr (43 hr after IVM) to age the oocytes. After aging, enucleated oocytes were removed from the incubator and exposed to 3 hr of 12°C. A single blastomere derived from an in vitro–produced morula at day 6 after IVF was injected into the perivitelline space of the enucleated oocyte, and the resulting couplet was placed in a 0.3 M mannitol solution containing 0.1 mM $MgSO_4$ and 0.05 mM $CaCl_2$ and exposed to a 1.5-kV electric pulse lasting 70 μsec. After electrical stimulation, oocytes were washed in TCM-199 and placed in MB2 with BOEC for culture.

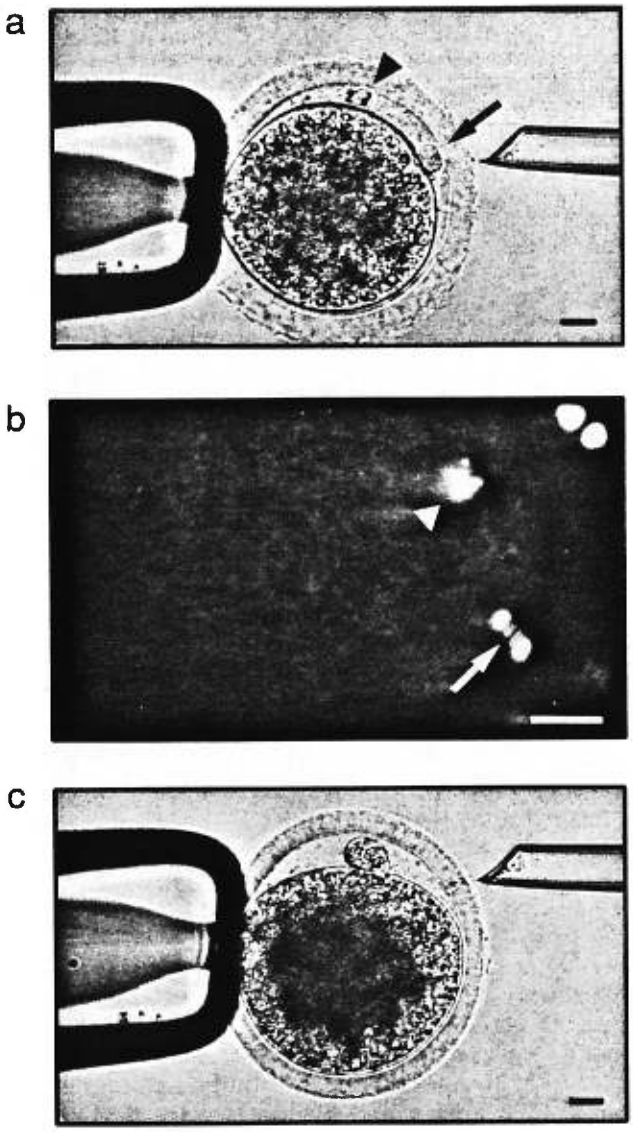

Fig. 1. Outline of the procedure for preparing activated telophase stage recipient oocytes for enucleation. **A:** Light photomicrograph of a telophase II oocyte at 3 hr after activation showing the degenerating first polar body separated from the oocyte membrane (arrowhead) and the second polar body closely attached to plasma membrane (arrow). **B:** Epifluorescence of the oocyte at 3 hr after activation stained with Hoechst 33342 and FITC antibody against α-tubuline showing the spindle connecting the second polar body in the perivitelline space and the spindle between the chromatin and the second polar body (arrow) and the fragmented chomatin present in the first polar body (arrowhead). **C:** Oocyte enucleated at telophase with a morula-stage donor blastomere in the perivitelline space. Note the reduced perivitelline space resulting from the removal of a small volume of cytoplasm with this procedure. Bars correspond to 15 μm.

In the second reconstruction protocol ("telephase" group; Fig. 1), oocytes were denuded of cumulus cells at 30 hr after IVM, exposed to an activation treatment, and returned to the incubator for 2 hr to allow for

TABLE 1. Activation Rate (Pronuclear Formation) of 26- and 30-hr Matured Oocytes at 12 hr after Exposure to Activation Treatments

Activation treatments	Activated at 26 hr		Activated at 30 hr	
	No. oocytes	% activated	No. oocytes	% activated
Control	60	8.1^{a}	61	$10.3^{a,b}$
RT	60	$13.4^{a,b}$	60	$22.1^{a,b,c}$
T-4	61	$28.0^{a,b,c}$	57	$47.1^{b,c,d,e}$
Ethanol	88	$57.2^{d,e}$	87	81.6^{f}
Ethanol + RT	58	$53.5^{c,d,e}$	89	$65.5^{e,f}$
Ethanol + T-4	70	$63.3^{d,e,f}$	74	$74.8^{e,f}$

Note: Different superscript denote significant differences ($P < 0.05$). RT, 3 hr exposure to room temperature (25°C); T-4, 3 hr exposure to 4°C; Eth, 5 min exposure to 7% ethanol.

extrusion of the second polar body (Powell and Barnes, 1992). After polar body extrusion, oocytes were exposed for 15 min to medium with cytochalasin B, and approximately a tenth of the cytoplasm surrounding the second polar body was removed. A blastomere obtained from a day 5.5 IVF-produced morula was introduced immediately into the perivitelline space, and the resulting couplet was exposed to an electric pulse as described above. Fusion between nuclear donor blastomeres and enucleated oocytes was verified visually, and the reconstructed oocytes were cultured in vitro for further 7 days to assess development to the blastocyst stage and blastocyst cell number by fixation and staining with Hoechst 33342.

Statistical Analysis

Frequencies of pronuclear formation among replicates were transformed by arcsine and square root to normalize the data for analysis of variance to enable treatment comparisons. Means were compared with the Tukey-Kramer HSD test, with the significance level set at 5%. Fusion and blastocyst-stage development rates were compared by chi-square and the number of cells per blastocysts by Student's *t*-test.

RESULTS

Characterizing an Effective Oocyte Activation Protocol

Our first objective was to test oocyte activation protocols using oocytes matured in vitro for 26 and 30 hr (Table 1). A total of 825 oocytes were utilized in 7 experimental replicates. In general, oocytes exposed to activation stimuli showed on average a higher ability to activate at 30 hr than at 26 hr from IVM oocytes (61.3% versus 44.3%, $P < 0.02$). Exposure of oocytes for 3 hr to room temperature (RT) and to 4°C (T-4) resulted in low activation rates when compared with the ethanol (Eth) and ethanol plus temperature-associated groups, both at 26 and 30 hr of IVM. However, oocytes exposed to 4°C at 30 hr differed only from the ethanol at 30 hr group, indicating that temperature reduction to 4°C is partially effective as a means for activating oocytes. The highest rates of pronuclear formation were obtained at

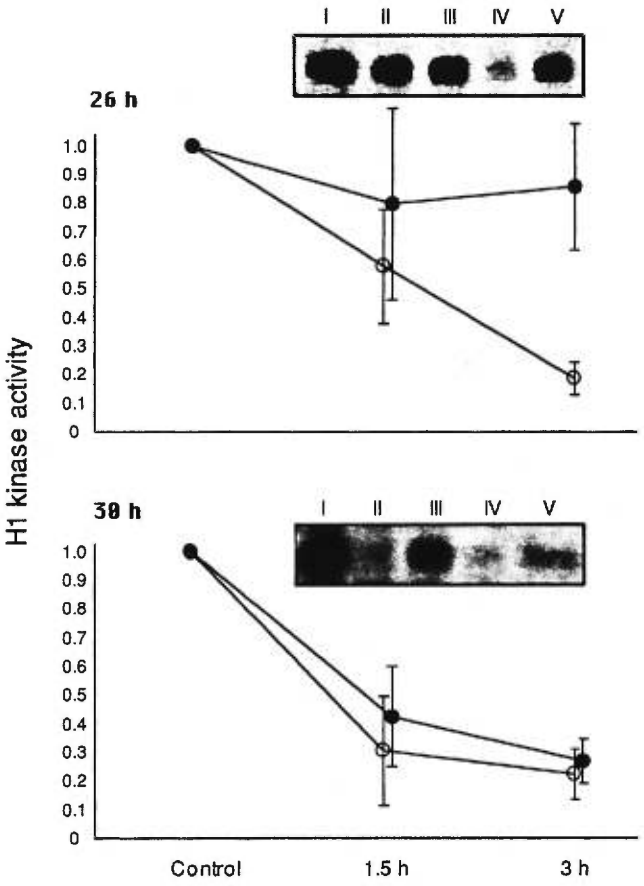

Fig. 2. Histone H1 kinase activity in control and activated oocytes matured in vitro for 26 and 30 hr. Oocytes were exposed to 7% ethanol for 5 min followed (close circle) or not (open circle) by a 3-hr cooling period at 4°C. Values are expressed as average relative intensity of the densitometric readings, and vertical bars indicate standard errors from four experiments. Inset image shows a representative autoradiograph of an H1 kinase assay of oocytes sampled before activation (control, I), 1.5 hr after activation with (III) or without (II) cooling, and 3 hr after activation with (V) or without (IV) cooling.

30 hr of IVM with oocytes exposed to ethanol alone or in conjunction with exposure to low temperatures (Eth + RT and Eth + T-4) and at 26 hr of IVM with oocytes exposed to Eth + T-4 ($P < 0.05$).

A further objective was to characterize the levels of histone H1 kinase activity in the first 3 hr following exposure to the activation stimuli that showed highest rates of pronuclear formation, thus ethanol and ethanol + T-4 (Fig. 2). A total of 250 oocytes at each 26 or 30 hr from IVM were exposed to these treatments and analyzed in groups of 10 oocytes for H1 kinase assay at either 1.5 or 3 hr after activation. Previous reports have indicated that most changes in MPF activity occur within a short period from activation or fertilization (Collas et al., 1993). Nontreated control oocytes at 26 and 30 hr also were assayed and used as internal standards to measure relative changes after the activation stimulus. Whereas the H1 kinase activity was similar among 26 hr and the 30-hr controls (100% versus 95%; $P > 0.05$), oocytes activated at 30 hr from IVM

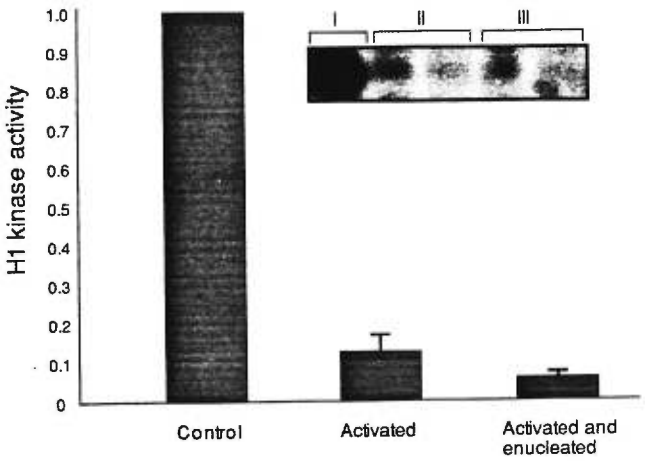

Fig. 3. Relative histone H1 kinase activity of control and telophase-enucleated oocytes manipulated at 36 hr from maturation. Columns represent the average densitometric reading of the H1 kinase bands and standard errors (vertical bars) from four experiments using 10 oocytes per band. Inset image shows a representative autoradiograph showing the nonactivated control (I), duplicates of the nonenucleated (II) and enucleated (III) groups activated at 32 hr and assessed at 36 hr from maturation.

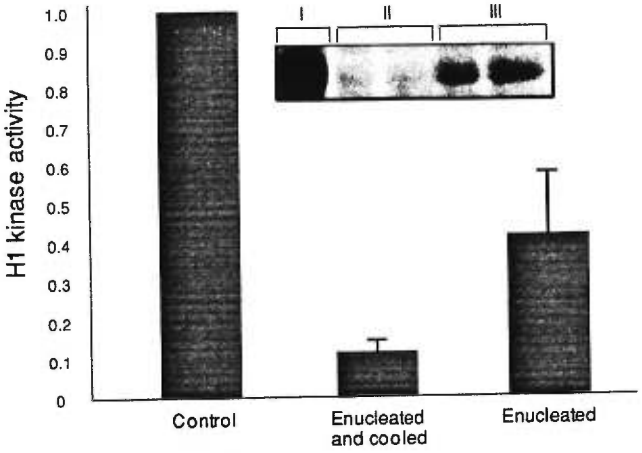

Fig. 4. Relative histone H1 kinase activity of control and aged-enucleated oocytes manipulated at 48 hr from maturation. Columns represent the average densitometric reading of the H1 kinase bands and standard errors (vertical lines) from four experiments using 10 oocytes per band. Inset image shows a representative autoradiograph showing the nonactivated control aged oocyte (I), duplicates of the enucleated cooled (II) and noncooled (III) groups. In this experiment cooling was performed at 12°C between 42 and 45 hr from maturation.

and kept at 39°C had lower levels of H1 kinase at 1.5 hr after treatment than the 26-hr group. Since no group difference was observed at 3 hr after treatment, these results indicate that a faster decay of kinase activity was present at 30 hr than in the 26-hr group. Oocytes cooled to 4°C immediately after ethanol treatment showed a less pronounced drop in H1 kinase activity at 26 and 30 hr. This was particularly evident in the 26-hr group, where H1 kinase levels remained practically unchanged after stimulation with ethanol. Since two-thirds of the oocytes in the 26-hr group formed pronuclei after this treatment (Eth + T-4), these results indicate that storage at 4°C may temporarily inhibit or slow the degradation of the H1 kinase activity until return to 39°C. Together these results show that ethanol alone is an effective procedure for activating oocytes and that optimal histone H1 kinase activity is attained within 1.5 hr of activation in oocytes at 30 hr from IVM.

Comparison of Aged and Telophase Oocyte Reconstruction Procedures

Once a suitable activation procedure was established, we were able to compare the "telophase" technique with the standard nuclear transfer "aged" technique used routinely to clone bovine embryos. A preliminary experiment was performed by fixing and staining 118 micromanipulated oocytes for DNA to determine the ideal time to enucleate activated oocytes. No difference in enucleation rates was observed when approximately 10% of the oocyte's cytoplasm with the extruding second polar body was removed by microsurgery. Chromatin removal at the telophase stage was equally obtained at 3 hr (97%), 4 hr (98%), or 5 hr (92%) after exposure to ethanol ($P > 0.05$). These results contrast with the 59% ($n = 752$) success rate obtained when enucleations were performed at the metaphase stage, when approximately 30% of the cytoplasm close to the first polar body is removed.

The activity of histone H1 kinase in the enucleated oocytes from both techniques was assessed for comparison. At 36 hr from IVM, the time at which fusion to the nuclear donor blastomere is normally performed, H1 kinase activity was at its minimum in activated and "telophase" enucleated oocytes, respectively, 12.4% and 5.7% of the levels found in nonmanipulated control oocytes at 36 hr from IVM (Fig. 3). Enucleated oocytes that were "aged" and cooled to 12°C for 3 hr prior to nuclear transfer had 11.5% of the H1 kinase activity of the nonmanipulated controls at 48 hr from IVM (Fig. 4). In contrast, enucleated oocytes "aged" but not cooled showed 42% of the H1 kinase activity observed in the 48 hr from IVM controls. Together, as long as the "aged" technique is preceded by cooling of the enucleated oocytes, these results indicate that both methods provide enucleated oocytes with similar levels of H1 kinase activity.

Our final objective was to compare the developmental competence of reconstructed oocytes produced using the "telophase" and "aged" techniques (Table 2). Although fusion rates did not differ among the groups, development of successfully fused oocytes to the blastocyst stage after 7 days of in vitro culture was significantly higher in the "telophase" group ($P < 0.001$). Moreover, blastocysts from the "telophase" group had significantly more cells than those from the "aged" group ($P < 0.02$). Comparisons with control IVF embryos produced in parallel with these experiments show no difference in potential to develop to the blastocyst stage nor in the number of cells per blastocyst, indicating that the "telophase" technique is as good as our standard in vitro embryo production technique.

TABLE 2. In Vitro Development of Reconstructed Embryos Using Telophase II and Aged Recipient Cytoplasts

Experimental group	No. of oocytes	No. of replicates	Fusion (%)	Blastocyst (%)	No. of nuclei per blastocyst (SE)
Telophase II	215	6	129 (58.1)	49 (38.0)[a]	126.2 (10.49)[c]
Aged	248	6	151 (59.7)	24 (16.0)[b]	83.7 (8.69)[d]

Note: Different superscripts within columns denote significant differences ([a,b]$P < 0.001$; [c,d]$P < 0.02$).

DISCUSSION

We hereby describe a method of enucleating bovine oocytes for use in nuclear transfer based on the removal of host chromatin after activation, at telophase stage of the second meiotic division. This method enables efficient enucleation with minimal removal of cytoplasm and avoids exposure to DNA stain or UV irradiation for chromatin localization. Moreover, enucleation at the telophase stage enables selection of a highly homogeneous group of synchronously activated host oocytes. Compared with oocytes enucleated at metaphase and aged before reconstruction, telophase-reconstructed oocytes supported the development of more blastocysts with a larger number of cells at day 7 after nuclear transfer.

Compared with ethanol alone, the association of ethanol and temperature reduction did not improve oocyte activation rates at either 26 or 30 hr from maturation, suggesting a nonsynergistic effect of these activation stimuli. Ethanol, like most other artificial stimuli, acts by inducing a single large transient increase of cytosolic calcium (Shiina et al., 1993) but lacks the long-lasting calcium oscillation pattern observed after fertilization (Fissore et al., 1992; Kline and Kline, 1992). Temperature reduction, on the other hand, has been shown to induce repeated calcium oscillations in a significant proportion of nonaged rat oocytes (Ben-Yosef et al., 1995). Together, the immediate calcium peak induced by ethanol and the continuous oscillations caused by temperature reduction should be complementary in promoting higher rates of pronuclear formation than either alone. Nonetheless, such synergistic effect was not observed in our studies, suggesting that reduced temperature may cause noneffective oscillations, as demonstrated in rats where the amplitude of the transients were two to three times lower in cooled oocytes (Ben-Yosef et al., 1995). Moreover, it is tempting to speculate that temperature reduction inhibits the complete degradation of CSF, causing an intermittent decrease in MPF levels and reentry into a third metaphase arrest, as observed in several mammalian species including cattle (Kubiak, 1989; Powell and Barnes, 1992). The latter is supported by previous reports showing that although nonaged oocytes revert to a metaphase-like state after initial activation (Collas et al., 1993, 1995), activation of aged oocytes is irreversible (Barnes et al., 1993). This may be due to low synthesis of the *c-mos* proto-oncogene product Mos (Wu et al., 1997), a putative active component of CSF (Lorca et al., 1993). Indeed, our results indicate that histone H1 kinase activity remained high for the 3 hr following activation of the 26-hr group, whereas noncooled oocytes showed basal H1 kinase levels at the same time. Regardless of cooling, however, 30-hr oocytes appear to show a more prompt decay in H1 kinase activity by 1.5 hr after ethanol exposure, suggesting a greater instability of MPF at this stage.

Low levels of MPF activity in recipient oocytes are essential to avoid the premature chromosome condensation (PCC) of interphase-stage donor nuclei during reconstruction (Collas and Robl, 1991; Szöllösi et al., 1988). Since oocytes show increasingly lower levels of MPF activity and can be more readily activated after prolonged periods of maturation, oocyte aging has been used widely as a means for obtaining suitable cytoplast for nuclear transfers (Ectors et al., 1995; Heyman et al., 1994; Stice et al., 1994; Zakhartchenko et al., 1995). The latter is confirmed by our findings and those from previous studies (Gall et al., 1996) showing that the cooling of enucleated oocytes after 48 hr of maturation leads to further decreases in the levels of H1 kinase activity when compared with the noncooled enucleated group. Gall et al. (1996) further proposed that enucleated oocytes obtained by aging and cooling are in a preactivated state in which low MPF and high MAP kinase activity may provide optimal conditions for the reprogramming of donor nuclei. However, oocyte aging has been shown to decrease viability for both in vitro fertilization (Chian et al., 1992) and after nuclear transfer (Du et al., 1995; Kono et al., 1994), suggesting that prolonged culture of secondary oocytes may affect crucial cytoplasmic constituents required for normal development.

Apart from the effects of aging, there are other steps involved in the enucleation of metaphase-arrested oocytes that are potentially harmful to the development of reconstructed embryos. First, metaphase spindles are often removed blindly during aspiration of a portion cytoplasm surrounding the first polar body. Since polar bodies may be located far from the original point of extrusion by the time of enucleation, a substantial amount of cytoplasm needs to be removed in order to achieve reasonable success. In our study, approximately a third of the total cytoplasmic volume was removed to achieve a 60% enucleation of metaphase oocytes. On the other hand, enucleation at the telophase stage relies on a precise positioning of the chromatin as it is being expelled in the second polar body, enabling successful enucleation of practically all manipulated oocytes with minimal removal of cyto-

plasm. Moreover, due to the high efficiency of the telophase technique, UV irradiation and Hoechst 33342 staining are not required to verify removal after enucleation. Both cytoplasm depletion (Evsikov et al., 1990; Westhusin et al., 1996) and UV stain exposure (Smith, 1993) have been reported to affect development, further supporting the advantages of an enucleation protocol that avoids these procedures. Finally, since it is rare that all the young oocytes in a sample are activated with artificial stimuli, several metaphase-enucleated oocytes fail to develop due to an inability to activate after stimulation. Telophase enucleation, on the other hand, enables the selection of oocytes that respond promptly to activation by extruding a second polar body within 2 hr of stimulation.

Together, when compared with the routinely employed metaphase enucleation approach using aged and cooled oocytes, the telophase approach showed improved potential to obtain blastocyst-stage embryos that had a larger number of cells. Although it is not possible to determine which elements of the protocol resulted in beneficial developmental effects, we believe that the proposed protocol enables the production of precisely synchronous enucleated cytoplasts that show an excellent development potential after fusion with morula-stage blastomeres. The use of synchronized donor nuclei may further improve the development of bovine oocytes reconstructed using this procedure.

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Article 2

(En préparation)

Ultraviolet-irradiated spermatozoa activate oocytes but arrest preimplantation development after fertilization and nuclear transplantation in cattle

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Abstract

Artificial means of oocyte activation are believed to lack an essential sperm epigenetic component required for early development. The main goal of this study was to examine the potential of ultraviolet-irradiated sperm as means of functionally eliminating the chromatin component of spermatozoa without affecting its ability to induce activation and support parthenogenetic development in cattle. Spermatozoa were stained with a bisbenzimide dye (Hoechst 3334210) for 10 min and exposed to different doses (10, 30 or 50 mJ/cm^2) of ultraviolet (UV) irradiation. Secondary oocytes were inseminated and either fixed after 18 h or cultured for a further 7 days to assess their ability to develop in vitro. Cleavage and blastocyst formation were significantly ($P<0.05$) reduced in all doses of UV-irradiation (10, 30 or 50 mJ/cm^2) and the higher doses of UV (30 and 50 mJ/cm^2) did also affect fertilization and pronuclear formation. After 18 h of insemination, sperm exposed to 10 mJ/cm^2 induced pronuclear formation at a rate similarly to non-treated controls (78.3 vs. 93.4%, $P > 0.05$). However, significantly fewer pronuclei were observed when the insemination period was reduced to 4 h (56.2 vs 85.2%, $P<0.05$). These results suggest that 10 mJ/cm^2 of UV-irradiation can functionally destroy the genomic component of spermatozoa with limited effects on its ability to induce oocyte activation. To confirm this preliminary conclusion, oocytes activated with UV-treated (Group 1; 10 mJ/cm^2) and untreated sperm (Group 2) were used as cytoplasm hosts in nuclear transfer and compared to parthenogenetically activated oocytes (Group 3). Developmental rates to cleavage stage were 57.1, 92.1 and 68.4%, and to blastocyst stage were 12.1, 36.5 and 27.0% for groups 1, 2 and 3, respectively, indicating that UV-treated spermatozoa blocked development even in the presence of a diploid donor nucleus. Although DNA replication, as measured by BrdU incorporation at the pronuclear stage, was not inhibited by UV-irradiation treatment, abnormal chromatin

conformations after cleavage suggest that sperm UV-derived DNA damage led to improper segregation of chromatin to daughter blastomeres during first mitotic division. Together, these results indicate that UV-irradiated sperm can activate oocytes but causes a developmental block at or soon after the first mitosis that precludes its use as a protocol for functional enucleation in nuclear transfer.

Introduction

A common approach to improve the developmental potential of embryos reconstructed by nuclear transfer is to pre-activate host oocytes in order to reduce the levels of the maturation-promoting factor (MPF), the kinase responsible for nuclear envelope breakdown and chromatin condensation in both meiotic and mitotic cells (Norbury, Nurse, 1992). Host cytoplasts with low MPF prevent the premature condensation and pulverization of blastomere donor chromatin that are frequently undergoing S-phase during early embryogenesis (Campbell et al. 1996). Artificial means of activating oocytes are more effective when using oocytes after prolonged metaphase II arrest, i.e. aged oocytes are more easily activated than when recently matured (Nagai, 1987; Ware et al. 1989; Kubiak, 1989). However, development potential after IVF (Chian et al. 1992) and nuclear transfer (Kono et al. 1994; Bordignon, Smith, 1998) is compromised by oocyte aging.

Artificial agents employed to activate oocytes are mostly unable to mimic the pattern of calcium oscillation induced by the sperm at fertilization (Kline, Kline, 1992; Sun et al. 1992; Fissore, Robl, 1993). It is well established that activation induced by sperm penetration involves a series of oscillations in the intracellular free calcium that persist for several minutes or even hours after fertilization, leading to MPF degradation, meiotic resumption and passage to interphase (Kline, Kline,

1992; Sun et al. 1992). Contrary to a single stimulus, repetitive calcium spikes induced by artificial means lead to a prompt and stable degradation MPF in different species (Ozil, 1990; Collas et al. 1993), indicating that calcium oscillations are required for an effective activation. Furthermore, it was shown that the calcium releasing agent introduced by the fertilizing sperm becomes associated with the nucleus up to the 2-cell stage and remains able to induce calcium oscillations, meiotic completion and pronuclear formation when transfered back to non fertilized oocytes (Kono et al. 1995). These results suggest that a non-genetic component of sperm is incorporated to the fertilized egg and plays an important role during the beginning of embryonic development. Moreover, studies indicated that a protein localized at the equatorial region of the spermatozoa is introduced into the oocyte at fertilization and may be the agent responsible for inducing intracellular calcium oscillations (Parrington et al. 1996), evidencing that oocyte activation is not dependent of chromatin integrity.

Spermatozoa DNA ablation through UV-irradiation has been successfully used for gynogenesis and ploidy manipulation in amphibian (Tompkins, 1978; Reinschmidt et al. 1979) and aquatics species (Streisinger et al. 1981; Chourrout, 1982; Malison et al. 1993; Don, Avtalion, 1993). In these species, UV-irradiation causes the complete inactivation of sperm chromatin without affecting its capability to penetrate or activate the oocyte. UV-radiation is preferentially used among other methods to induce sperm chromatin ablation due to reduced DNA fragmentation (Chourrout, 1984; Chourrout, 1986). In fish, UV-irradiation has also been used to functionally enucleate eggs in androgenetic studies (Bongers et al. 1994; Lin, Dabrowski, 1998). In mammals, the effects of UV-irradiation has been evaluated in oocytes and eggs (Tsunoda et al. 1988; Smith, 1993; Bradshaw et al. 1995), and proposed as a potential mechanism for functional enucleation of recipients oocytes for nuclear transfer (Tsunoda et al. 1988; Bradshaw et al. 1995). However, attempts

to use UV-irradiation to destroy DNA as a means to functionally remove the genetic contribution of sperm has not been tested at present. If applicable, this procedure could be used not only for activating non-aged oocytes in nuclear transfer but also to assess the genetic and non-genetic contribution of sperm to embryonic development. Therefore, the main goals of this study were, first, to determine whether ultraviolet irradiation of sperm leads to chromatin ablation without affecting its capability to activate oocytes at fertilization and, second, to verify whether oocytes activated with UV-irradiated sperm can be used as recipient cytoplasts for nuclear transfer. We show that UV-exposed spermatozoa remain capable of penetrating and activating non-aged bovine oocytes without supporting develoment beyond the 2-cell stage. However, UV-irradiated sperm chromatin appears to participate in the formation of the first mitotic spindle and, thereby, interferes with the balanced segregation of chromatin to daughter blastomeres.

Materials and Methods

Oocyte and embryo source

Cow ovaries were obtained from a local abattoir, placed in saline at 35 °C, and transported to the laboratory within 2 h from slaughter. Cumulus-oocyte-complexes (COCs) were aspirated from 2-8 mm antral follicles using a 19-gauge needle and selected for the presence of several layers of cumulus cells and oocytes with homogeneous cytoplasm. Selected COCs were washed in HEPES-buffered tissue culture medium (TCM-199; Gibco BRL, Burlington, Canada) supplemented with 10% (v/v) fetal calf serum (FCS; Gibco). Groups of 20 COCs were matured for 24 h in 100 µl drops of TCM-199 supplemented with 10% of fetal calf serum (FCS; Gibco), 1 µg ml^{-1} estradiol-17ß (Sigma, St. Louis, MO), 50 µg ml^{-1} LH

(Ayerst, London, ON, Canada), 0.5 μg ml $^{-1}$ FSH (Folltropin-V; Vetrepharm, St. Laurent, Canada), 22 μg ml $^{-1}$ pyruvate (Sigma) and 50 μg ml $^{-1}$ gentamicin (Sigma) at 39 °C in 5% CO_2. In vitro fertilization was performed using the procedures described by Parrish et al. (1986). Briefly, expanded COCs were placed in 50 μl drops of Tyrode's medium, supplemented with 0.6% bovine serum albumin (BSA; Fraction V, Sigma), lactate, pyruvate, gentamicin, and 10 μg ml $^{-1}$ heparin. Frozen-thawed spermatozoa were washed and centrifuged through a gradient of Percoll (Pharmacia, Sweeden) and diluted at 10^6 live spermatozoa ml $^{-1}$. After 4 or 18 h from insemination, oocytes were denuded of cumulus cells by brief shaking, and transferred to 50 μl drops of Menezo's B2 medium (MB2; Pharmascience, Paris, France) supplemented with 10% FCS in the presence of bovine oviductal epithelial cells (BOEC). All the cultures were performed in drops under equilibrated mineral oil at 39 °C in a humidified atmosphere of 5% CO_2 in air.

Ultraviolet sperm treatment

Frozen-thawed spermatozoa were incubated for 15 min with 10 μg ml $^{-1}$ of Hoechst 33342 (Sigma), washed through a Percoll, adjusted for a final concentration of 10^6 live spermatozoa ml $^{-1}$, and placed in 50 μl drops covered with a thin layer (4 ml in a 35 mm diameter dish) of mineral oil (Sigma). Sperm were exposed to different doses of UV-radiation (10, 30 or 50 mJ/cm^2) using a ultraviolet chamber (GS Gene Linker Ultraviolet Chamber; Bio-Rad, CA). An average of 15 in vitro matured COCs were placed in sperm drops 1 h after UV-irradiation and cultured during either 4 or 18 h. Oocytes incubated with sperm during 4 h were denuded of cumulus cells and cultured for a further 12 h in fertilization medium in the absence of sperm whereas those incubated with sperm for 18 h were denuded of cumulus cells at the end of the insemination period. Both groups were either fixed immediately after removal from IVF drops or

cultured for a further 7 days to evaluate their ability to cleave (44 h) and develop to the blastocyst stage (day 7) in vitro.

DNA synthesis assay

Inseminated oocytes were incubated in the presence of 100 µM of bromodeoxyuridine (BrdU; 5-bromo-2'-deoxyuridine 5'-triphosphate, Sigma) to determine if the UV-irradiated sperm affected the pattern of DNA synthesis during pronuclear formation. After IVF using control (non-treated) or UV-irradiated (10 or 30 mJ/cm^2) spermatozoa, oocytes were denuded of cumulus cells and incubated in BrdU for different periods (4-8h, 4-18h, 8-16h or 18-24h). At the end of each incubation period, oocytes were fixed in formalin 10% for 10 min, permeabilized using 0.5% Triton X-100 (Biopharm, Laval, Canada) in PBS for 2 h and washed twice in block solution (PBS, 3% BSA, 0.1% Tween-20) for 20 min at room temperature. Oocytes were incubated for 1 h in 10 µl of anti-BrdU monoclonal antibody (Amersham, Oakville, Canada) containing 1 µg ml^{-1} DNAse, washed in block solution and incubated with the secondary antibody (fluorescein conjugated goat anti-mouse IgG, Sigma) at 1:100 dilution. Finally, oocytes were washed in block solution, mounted onto slides in Mowiol (Aldrich, Milwaukee, WI) containing 5 µg ml^{-1} of Hoechst 33342, and examined by epifluorescence using a filter block at 380 nm excitation and 420 nm emission (UV-2A; Nikon, Tokyo). Pronuclei of control and UV-irradiated groups were ranked according to the intensity of the emitted fluorescence.

Chromatin and spindle morphology assay

At 24 h after IVF, inseminated oocytes were fixed in formalin, permeabilized as described above and exposed to a primary antibody raised against mouse α-tubulin (Sigma) diluted 1:1000. The secondary antibody was similar to that used for the BrdU assay. Chromatin morphology was evaluated at the pronuclear (18 h) and 2-cell stages (30 h)

in both IVF and reconstructed embryos. Chromatin morphology and number of nuclei in blastocysts were obtained by mounting embryos onto a glass slide using Moviol containing 5 μg ml $^{-1}$ of Hoechst 33342 and epifluorescence examination.

Oocyte activation and reconstruction

Sperm-mediated oocyte activation was assessed at 4 h after insemination with either control or UV-irradiated (10 mJ/cm^2) sperm, and those presenting a second polar body were selected to be micromanipulated. Parthenogenetic activation was obtained by exposure of in vitro matured oocytes to 5 μM ionomycin (Sigma) for 4 minutes in TCM-199 HEPES-buffered medium, supplemented with 2 mg ml $^{-1}$ BSA, washed and cultured for an additional 2 h for second polar body extrusion. Activated oocytes were enucleated at telophase II stage by removing a small portion of cytoplasm surrounding the position of the second polar body, described previously (Bordignon, Smith, 1998). A single blastomere derived from an in vitro-produced morula at day 5 after IVF was injected into the perivitelline space of the enucleated oocyte. The resulting couplet was placed in a 0.3 M mannitol (Sigma) solution containing 0.1 mM $MgSO_4$ and 0.05 mM $CaCl_2$ and exposed to a 1.5-Kv electrical pulse lasting 70 μsec. After electrical stimulation, oocytes were washed in PBS and cultured in MB2 in the presence of BOEC. Fusion between nuclear donor blastomeres and enucleated oocytes was verified 1 h later and development to cleavage and blastocyst at 30 h and day 7, respectively. Androgenetic embryos were produced by fertilizing metaphase-stage enucleated oocytes. After 24 h of IVM, oocytes were denuded of cumulus cells and enucleated by removing approximately 30% of the cytoplasm surrounding the first polar body. After microsurgery, oocytes were placed in medium containing 5 μg ml $^{-1}$ of Hoechst 33342 for 15 min and exposed briefly to UV irradiation to verify the absence of chromatin. Enucleated oocytes were placed in the presence of UV-

irradiated (10 mJ/cm^2) or control sperm for 4 h, and fixed after 18 h (before cleavage) or 30 h (after cleavage), stained and examined by epifluorescence to assess chromatin morphology.

Statistical analysis

Percentage development to pronuclear, cleavage and blastocyst stages were arc-sine transformed and square-rooted to normalize the data. All data were analyzed by ANOVA and means were compared using Tuckey-Kramer test at 5% level of significance.

Results

Experiment 1: Effect of sperm UV-irradiation on oocyte activation and pronuclear formation

The first objective of this study was to determine whether ultraviolet-irradiated sperm had the ability to activate oocytes and, if so, to determine whether the irradiation of paternal chromatin would influence the developmental outcome after fertilization. Pronuclear formation, cleavage and blastocyst development percentages were assessed in oocytes cultured for 18 h in the presence of control and UV-treated sperm exposed to three different doses of irradiation (Table I). Sperm exposed to 10 mJ/cm^2 of UV-irradiation enabled pronuclear formation as often as non-irradiated controls ($P>0.05$). However, sperm exposed to higher doses (30 and 50 mJ/cm^2) showed a reduced fertilization potential ($P<0.05$) evidenced by a low percentage of pronuclear stage zygotes at the end of 18 h culture. Cleavage percentages to the 2-cell stage at 48 h post insemination was significantly reduced ($P<0.05$) at all UV doses examined, indicating blockage of the first cleavage division regardless of an apparently normal activation and pronuclear formation. Development to the blastocyst stage was completely inhibited with sperm exposed to 30

and 50 mJ/cm^2 of UV and only a small percentage of oocytes achieved the blastocyst stage when fertilized with sperm exposed to 10 mJ/cm^2.

A second group of oocytes was placed for a shorter period (4 h) in insemination drops containing control and irradiated sperm to enable a more precise timing of the fertilization event (Table II). At shorter inseminated periods, non-irradiated sperm led to significantly higher pronuclear formation than the irradiated groups ($P<0.05$). None-the-less, although cleavage to the 2-cell stage was severely compromised and the development to blastocyst stage almost completely inhibited, pronuclear formation after 10 mJ/cm^2 irradiation was observed in over half of the inseminated oocytes. These results indicate that one can achieve reasonable percentage of more precisely timed UV-exposed sperm-activated oocytes using low levels of UV-irradiation. However, levels of pronuclear formation after fertilization with sperm exposed to 30 mJ/cm^2 of UV was remarkably low, precluding its possible use for the reconstruction of oocytes activated with UV-treated sperm.

Experiment 2: Embryo reconstructions using oocytes activated with UV-irradiated sperm

Our objective in this study was to verify whether oocytes activated with UV-irradiated spermatozoa could be used as host cytoplasts in the reconstruction of oocytes by nuclear transplantation. Host oocytes in control groups were activated either artificially (ionomycin) or using non irradiated sperm (control sperm) and enucleated at telophase (Table III). The developmental rate both to cleavage and blastocyst stage was significantly ($P<0.05$) reduced when using recipient oocytes activated with UV-irradiated sperm, indicating that the presence of UV-irradiated sperm chromatin was detrimental to development regardless of the presence of a normal diploid donor nucleus. High yields of embryos were obtained with reconstructions using host oocytes activated with non irradiated sperm, indicating that triploidy does not affect their developmental outcome. The

number of nuclei per blastocyst at day 7 of culture was similar among treated and control groups.

Experiment 3: DNA synthesis and chromatin morphology assay

Our final goal in the present study was to identify the possible causes for the cleavage and developmental blockage observed in oocytes after activation with UV-irradiated spermatozoa. Initially, DNA synthesis patterns of the resulting male pronucleus was measured using a BrdU uptake assay. Assays were performed at different periods throughout the first cell cycle, i.e. from 4 to 8, 8 to 16, 18 to 24 and 4 to 18 h after insemination with control and 10 or 30 mJ UV-irradiated sperm (Fig. 1). Although a weaker BrdU staining was observed during the period from 4 to 18 h post insemination in UV-irradiated groups ($P<0.05$), all male pronuclei stained positively for BrdU, indicating that DNA replication occurred regardless of the irradiation of spermatozoa. Compared to UV-irradiated groups, stronger BrdU staining was seen in the control group during the first few hours after insemination (from 4 to 8 h), suggesting that fertilization occurred later in the UV-irradiated sperm. However, no significant difference in BrdU incorporation was seen during the period of major DNA replication (8 to 16 from IVF) or at the end of the first cell cycle from 18 to 24 h post insemination ($P>0.05$).

To evaluate the morphology of pronuclei at the end of the first cell cycle, chromatin was observed by epi-fluorescence at 18 h post insemination. Most of androgenetic embryos produced by fertilization of enucleated oocytes using UV-irradiated spermatozoa showed smaller pronuclei with condensed and pulverized chromatin (Fig. 2). None of these abnormalities were observed in androgenetic embryos produced with non irradiated sperm. However, the pronuclear morphology of both inseminated and reconstructed embryos produced with UV-irradiated sperm was apparently not affected (Fig. 3). Most embryos in the latter groups possessed two well-developed pronuclei with decondensed

chromatin. Conversely to fertilized and nuclear transfer embryos, androgenetic groups derived of UV-irradiated spermatozoa showed damaged chromatin when analyzed as 2-cells at 30 h after insemination. The abnormalities seen included chromatin fragmentation, consisting of threads of chromatin bridging sister blastomeres (Figs 3e and 3f) and pulverized and picnotic condensed chromatin in nuclear transfer and IVF groups, and a completely degenerated chromatin in the androgenetic group (Figs. 2c and 2d). Whereas all 2-cell stage androgenetic embryos contained abnormal chromatin, non-enucleated oocytes fertilized with 10 and 30 mJ/cm^2 UV-irradiated sperm showed abnormal chromatin in 67% and 76%, respectively. Most of oocytes fertilized with UV-treated sperm stopped development at 2-cells stage, since 57.1 % (n=28) of oocytes fertilized with UV-irradiated sperm remained at 2-cells at 50 h post IVF compared to only 7.7% (n=52) of those in the control group. Moreover, only 10.7 % had achieved the 8-cells stage compared with 71.1% in the control group. These results show that UV-irradiated sperm is decondensed after penetrating the oocyte and develops a DNA synthesizing pronucleus that participates in the singamy. None-the-less, most embryos arrest development either before or soon after first cleavage. Although only a small number of oocytes were fixed at mitosis, no apparent abnormality was seen in the alignment of chromatin onto the metaphase plate, suggesting that spindle formation is not directly involved in the abnormalities observed at the 2-cells stage.

Discussion

The main goal of the present study was to establish a procedure to induce ablation of sperm chromatin without inhibiting the capacity of spermatozoa to penetrate and activate metaphase arrested oocytes. We show that the capacity of UV-irradiated spermatozoa to penetrate and

activate oocytes is dependent on the dose of UV applied. Whereas lower doses did not reduce the fertilizing ability of sperm, development to the blastocyst stage was almost completely blocked by UV-irradiation, suggesting functional destruction of the chromatin. Transplantation of morula-derived blastomere nuclei into oocytes fertilized with UV-irradiated sperm leads to a partial recovery of development to the blastocyst stage. Morphological evaluations by DNA staining suggest that the inhibition of development may have been caused by chromatin fragmentation induced by UV-irradiation.

Effect of sperm UV-irradiation on oocyte activation

Results from this experiment show that the fertilization capacity of bovine spermatozoa exposed to UV-irradiation is dependent on the dose applied. Although UV-irradiation of sperm had no apparent effect on motility immediately after exposure, only sperm exposed to lower doses of irradiation were able to penetrate and activate in vitro matured oocytes at a rate similar to non irradiated sperm. Moreover, high doses of radiation (30 and 50 mJ/cm^2) led to significantly lower fertilization rates. The chromatic component of sperm is not considered to be involved in the activation (Parrington et al. 1996; Fissore et al. 1998), suggesting that UV-effects on chromatin are unlikely to affect fertilization rates. However, apart from effects on chromatin, exposure to UV may affect other sperm components such as mitochondria or membranes integrity, as evidenced after the irradiation of bovine oocytes (Smith, 1993) and fish spermatozoa (Don, Avtalion, 1993).

Although lower doses of UV did not affect the activation and penetration rates of spermatozoa, development to the 2-cell (cleavage) and blastocyst stages was seriously compromised at low and high levels of UV-irradiation. These results contrast with those described in fish and amphibian where intermediate doses of UV led to minimum embryonic survival and a recovery of development potential was observed with the

use of higher doses. It was concluded that intermediate doses of irradiation cause an incomplete destruction of the chromatin allowing fragments of sperm chromatin to be incorporated into the maternal chromatin leading to improper expression of the paternal genes, and interfering with the embryonic development (Chourrout, 1982; Chourrout, 1986; Don, Avtalion, 1993). Conversely, lower doses do not cause significant damages to chromatin and higher doses can completely inhibit sperm chromatin allowing the development of the gynogenetics embryos. Surprisingly this effect was not seen in the present study since higher UV doses reduced drastically the fertilization rate and no improvements to embryonic development were observed in the few fertilized oocytes. In contrast to the rapid development of fish and amphibian early embryos, the first cell cycle is much longer in bovine embryos, suggesting that the damage caused to chromatin could be partially repaired by nucleotide excision repair mechanism operating into the oocytes (Masui, Pederson, 1975; Generoso et al. 1979; Brandriff, Pedersen, 1981). Therefore, partially repaired chromatin would reverse the effect of UV and thereby cause a negative influence on subsequent development. When the time of exposure of irradiated sperm was reduced to 4 h, the overall effects of irradiation was similar to the 18 h period. However, a significant reduction in fertilization rate was observed when a lower doses of radiation was applied, indicating that UV-irradiated sperm require a longer period to penetrate and activate the oocytes when compared to non irradiated controls.

Nuclear transfer

Our objective in this study was to determine whether oocytes fertilized with UV-irradiated sperm and fused to a non-irradiated diploid nucleus from a morula-stage blastomere can develop in vitro. Our results show that the development of reconstructed embryos using hosts cytoplast activated with UV-irradiated (10mJ/cm^2) spermatozoa was

significantly lower than reconstructed oocytes activated with non-irradiated sperm or parthenogeneticaly (table III), indicating that the harmful effect of UV-irradiated sperm on embryo development persisted after the transfer of a diploid nucleus. However, compared with the results obtained after fertilization using sperm exposed to the same dose of UV (table I and II), more embryos cleaved and developed to the blastocyst stage after nuclear transplantation, suggesting that the transfer of a diploid nucleus removed at least partially the inhibitory effect of UV-irradiated sperm. The transmission of a viable centrosomal pair with the diploid nucleus may have enabled a better developmental outcome. In bovine, the establishment of the centrosomal component required for normal spindle assembly seems to be of paternal and maternal origin and that either one or two centrosomes are carried into the host oocyte after the transfer of a morula derived blastomere (Navara et al. 1994). Therefore, since UV-irradiation may affect the spermatic centrosome and lead to low cleavage, the introduction of normal centrosomes with the transferred blastomere would improve cleavage rates in reconstructed embryos. On the other hand, high cleavage and blastocyst development has been reported in parthenogenetic embryos in different species (Susko-Parrish et al. 1994; Loi et al. 1998), suggesting that the paternal centrosome may not be absolutely required for cleavage.

The effect of UV on the chromatin

The final objective was to determine a possible cause of the embryonic development blockage occurring after fertilization with UV-irradiated spermatozoa. Patterns of DNA synthesis during the first cell cycle was assessed by BrdU incorporation to determine whether DNA replication was affected by UV-irradiation. Our results show that, independently of UV-irradiation, DNA replication occurred in all zygotes analyzed, indicating that sperm chromatin was not completely damaged by UV-exposure. However, the pattern of BrdU incorporation was

significantly reduced by UV-irradiation, indicating a low synthesis of DNA. A similar effect of UV-irradiation on the reduction of BrdU uptake was previously reported using cultured human fibroblasts (de Laat et al. 1996). This effect may be due to a physical blockage of replication fork movement by UV-induced lesions on the chromatin (Moore et al. 1981). Further than a general reduction in the BrdU uptake seen during the period of 4 to 18 h post fertilization, we have shown that oocytes fertilized with irradiated sperm have a reduced synthesis of DNA during the first 8 h post fertilization. A delay in the fertilization time or a slow remodeling of the sperm chromatin may have accounted for this effect. Indeed, as observed in hamster ovary cells synchronized at the G1-phase, UV-irradiation causes a dose dependent delay in entry into S-phase (Orren et al. 1995).

Concerning the effect of UV-irradiation on chromatin morphology, the most remarkable anomaly observed was the different patterns of chromatin fragmentation seen after the first cell division. A high proportion of the embryos showed dispersed fragments of chromatin forming small nuclei or threads of chromatin linking the nuclei of sister blastomeres, indicating that, despite of DNA damaged caused by UV-irradiation, sperm chromatin participated in the formation of the mitotic spindle. Surprisingly, no apparent morphological effect of UV-irradiation was seen in pronuclei during the first cell cycle after fertilization. However, androgenetic embryos derived of enucleated oocytes fertilized with UV-irradiated sperm had completely abnormal pronuclear morphology, suggesting that the presence of the intact female chromatin has a beneficial effect on the remodeling of irradiated sperm chromatin.

It is known that the main injury induced by UV-irradiation on the chromatin is the formation of cyclobutane dimmers, a covalent link between adjacent pyrimidine bases, which affect the chromatin activity, including DNA replication and transcription (Moore et al. 1981; Mitchell, 1988; Doetsch et al. 1995; Tommasi et al. 1996). Moreover, in response

to the chromatic damages induced by radiation, eukaryotic cells can activate regulatory pathways known as cell cycle checkpoints which control the order and timing of cell cycle transitions to ensure that critical events such as DNA replication and chromosome segregation are completed with high fidelity (Elledge, 1996). Although cells have a nucleotide excision repair mechanism used to remove and replace affected bases, and reverse the effect of radiation (Sancar, Sancar, 1988), spermatozoa are not provided with this mechanism and are unable to repair damages induced by radiation or chemicals (Chandley, Kofman-Alfaro, 1971; Kofman-Alfaro, Chandley, 1971; Gledhill, Darzynkiewicz, 1973). On the other hand, eggs have an excision-repair capacity (Masui, Pederson, 1975) which can also act on sperm DNA that has been damaged before fertilization (Generoso et al. 1979; Brandriff, Pedersen, 1981; Matsuda, Tobari, 1988). This mechanism combined with the long period required to complete the first cell cycle may have allowed the recovery of chromatin damages induced by UV and consequently would account for the inability to induce a complete DNA ablation after UV-irradiation.

Our results suggest that the inability of many embryos to cleave when fertilized with UV-irradiated sperm may have been caused by blockage at G2-phase since both pronuclei were shown to incorporate BrdU throughout the first cell cycle. Indeed, although DNA damaged checkpoints can block cells at different phases of cell cycle, including G1/S, S and G2/M (Paulovich et al. 1997), UV-irradiated cells seem to block more frequently at G2 (Orren et al. 1995; Gabrielli et al. 1997). The G2/M arrest checkpoint is probably caused by breaks in the chromatin, and consists of a period required for repairing DNA damages (Paulovich et al. 1997). Nevertheless, most cleaved embryos showed abnormal chromatin morphology, indicating that they were able to cleave regardless of damaged chromatin and suggesting that G2 cell cycle checkpoints may not be fully operational during early embryogenesis in cattle. Whenever the G2 arrest checkpoint fails, broken chromosomes can be partitioned

into separated nuclei, precluding the possibility of their undergoing end-to-end fusion, leading to a variety of outcomes, including degradation or formation of truncated chromosomes (Paulovich et al. 1997).

Although the exact molecular mechanism implicated in the arrest of the cell cycle in response to DNA damage remains unclear, the signal seems to depend on the p53 tumor repressor protein (Kastan et al. 1991; Dumaz et al. 1997) that can induce the transcription of several genes, some of which can arrest the cell cycle or even induce cell apoptosis (Elledge, 1996). However, the arrest of cells at G2 stage seems not to be dependent of p53 (Wang et al. 1996) and possibly involves a block in the cdc25-dependent activation of cyclin/cdc2 kinase (Poon et al. 1996; Gabrielli et al. 1997). Taking into account that a proportion of embryos were arrested before and others after the first cleavage, we may infer that different pathways are involved in the blockage of embryonic development after fertilization with UV-irradiated sperm.

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Table I. Percentage pronuclear formation, cleavage and blastocyst development of oocytes fertilized with UV-irradiated spermatozoa (IVF – 18 h).

Dose of UV	2 pronuclei % (N)	Cleavage % (N)	Blastocyst % (N)
Control	93.4 (135)^{a}	73.7 (161)^{a}	29.0 (161)^{a}
10 mJ/cm2	78.3 (105)^{a}	36.9 (147)^{b}	2.7 (147)^{b}
30 mJ/cm2	47.8 (132)^{b}	23.0 (190)^{bc}	none (190)^{b}
50 mJ/cm2	25.7 (92)^{b}	14.4 (116)^{c}	none (116)^{b}

Means within columns with different superscripts differ significantly ($P<0.05$).

Table II. Percentage pronuclear formation, cleavage and blastocyst development of oocytes fertilized with UV-irradiated spermatozoa (IVF – 4 h).

Dose of UV	2 pronuclei % (N)	Cleavage % (N)	Blastocyst % (N)
Control	85.2 (278)[a]	65.1 (570)[a]	30.7 (570)[a]
10 mJ/cm2	56.2 (296)[b]	19.0 (596)[b]	0.7 (596)[b]
30 mJ/cm2	31.3 (246)[c]	11.2 (466)[c]	none (466)[b]

Means within columns with different superscripts differ significantly ($P<0.05$).

Table III. Development of nuclear transfer embryos reconstructed using hosts cytoplasts activated artificially, control and UV irradiated spermatozoa.

Activation	No. Reconstructed	% Cleavage	% Blastocyst	No. Nuclei (SE)
Ionomycin	74	75.6^{a}	24.3^{a}	110±19
Control sperm	67	88.1^{b}	28.4^{a}	114±24
UV sperm	76	53.3^{c}	9.2^{b}	115±13

Means within columns with different superscripts differ significantly ($P<0.05$).

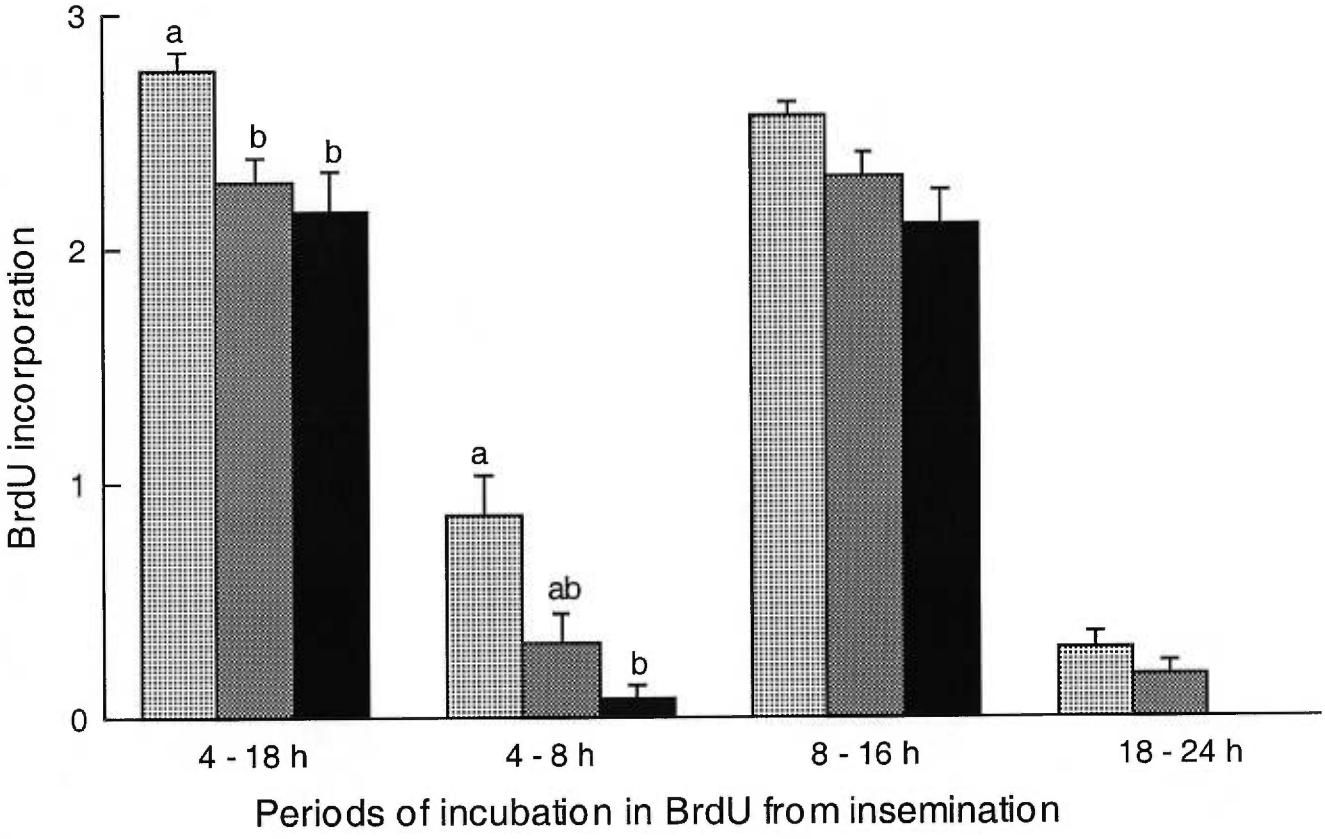
3
2
1
0
BrdU incorporation
a
b
b
a
ab
b
4 - 18 h
4 - 8 h
8 - 16 h
18 - 24 h
Periods of incubation in BrdU from insemination

Figure 1.

Patterns of BrdU incorporation in pronuclei of embryos fertilized with UV-irradiated sperm. Oocytes were placed into fertilization drops in the presence of control (light gray), 10 mJ/cm^2 (gray bar) and 30 mJ/cm^2 (black bar) UV-irradiated sperm for 4 h and incubated in the presence of BrdU for different periods after insemination (0 h). The intensity of BrdU staining was assessed subjectively in a total of 227 pronuclear stage zygotes (average 19 per group) and ranked into four categories: none (0), weak (1), medium (2) and strong (3). Vertical bar represent standard errors of the mean rankings for each group and different letters represent differences within periods ($P \leq 0.05$).

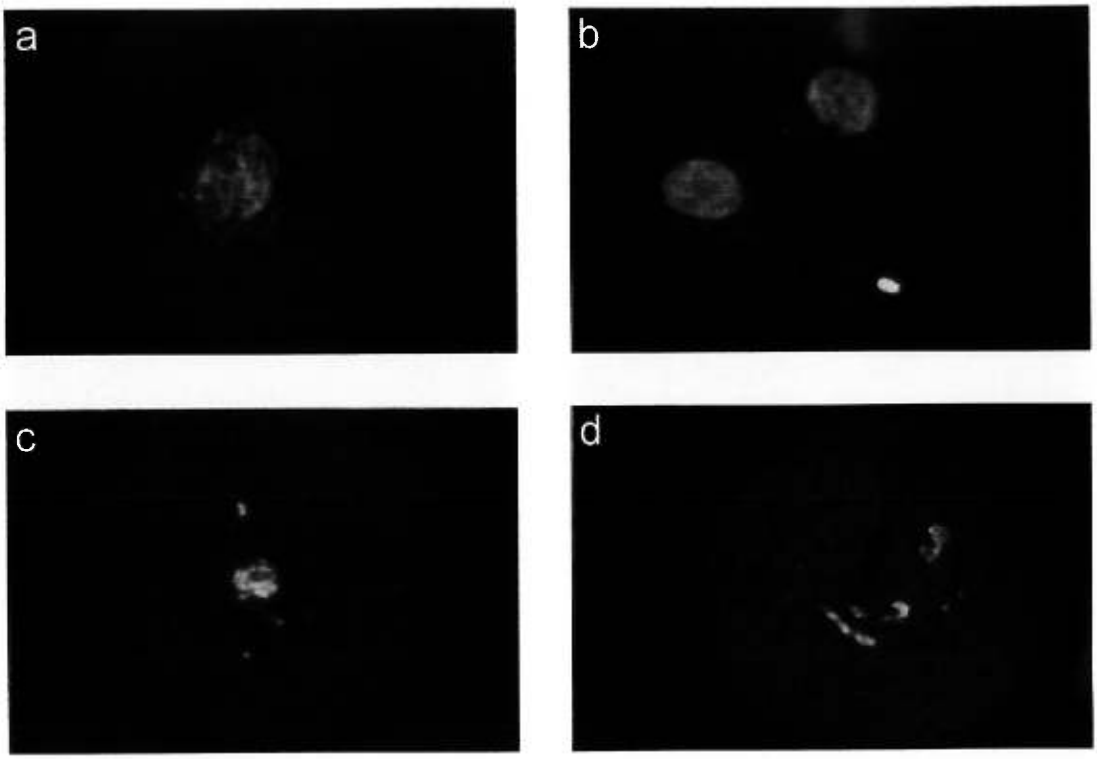
a
b
c
d

Figure 2.

Chromatin morphology of androgenetic embryos inseminated with control and UV-irradiated sperm. Oocytes were enucleated at metaphase II and inseminated with either non-irradiated sperm, and fixed at the pronuclear (a) and 2-cell (b) stages, or UV-irradiated sperm (10 mJ/cm^2), and fixed at the pronuclear (c), 2-cell (d) stages. Magnification 500 times.

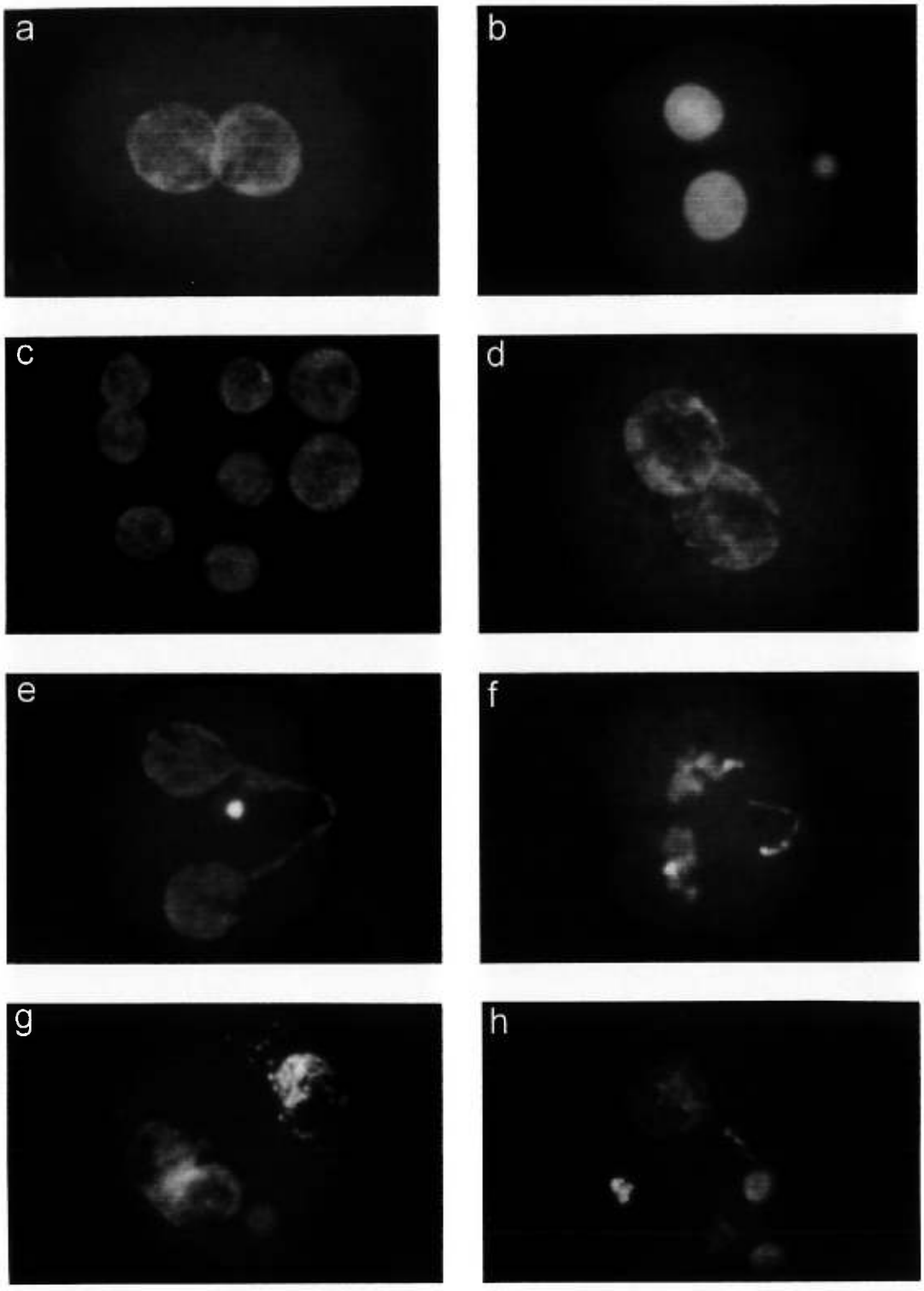
a
b
c
d
e
f
g
h

Figure 3.

Chromatin morphology of embryos inseminated with control and UV-irradiated sperm. Oocytes inseminated with non-irradiated sperm at the pronuclear (a), 2-cell (b) and 8-cell (c) stages. Oocytes inseminated with UV-irradiated (10 mJ/cm^2) at the pronuclear (d), 2-cell (e,f) and 4- to 8-cell (g,h) stages. Magnification 500 times.

Article 3

(Accepté - Biology of Reproduction)

Developmentally regulated loss and reappearance of immunoreactive somatic histone H1 on chromatin of bovine morula-stage nuclei following transplantation into oocytes[1]

Short title: Somatic histone H1 in nuclear-transplant embryos

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ABSTRACT

One difference between chromatin of bovine oocytes and blastomeres is that somatic subtypes of histone H1 are undetectable in oocytes and are assembled onto embryonic chromatin during the fourth cell cycle. Here, we have investigated whether this chromatin modification is reversed when nuclei containing somatic H1 are transplanted into ooplasts. Donor nuclei obtained from morula-stage bovine embryos were fused to ooplasts, at different times before and after parthenogenetic activation of the ooplasts. Following fusion, immunoreactive H1 became undetectable and the loss occurred more rapidly when fusion was performed near the time of ooplast activation as compared with several hours after activation when the host oocytes were at a stage corresponding to interphase. Although the loss of immunoreactive H1 occured independently of DNA replication and transcription, exposure of reconstructed oocytes to cycloheximide or 6-DMAP delayed the loss of immunoreactive H1 from transplanted nuclei. During further development of nuclear-transplant embryos, somatic H1 remained undetectable at the 2- and 4-cell stages, and re-appeared on the chromatin at the 8- to 16-cell stage, as previously observed in unmanipulated embryos. We conclude that factors in oocyte cytoplasm are able to modify morula chromatin so that somatic H1 becomes undetectable, and that the amount or activity of these factors declines over time in activated ooplasts.

INTRODUCTION

The fertilized egg gives rise to all the cells of a new organism, and its nucleus therefore is defined as totipotent. The cells that descend from the egg during embryonic development become progressively restricted, however, in the differentiation pathways that they can follow. It has long been questioned whether this loss of totipotency reflects irreversible changes in the nuclei of somatic cells, which would render large portions of the genome non-expressible. Classical studies performed in marine invertebrates and amphibians demonstrated that nuclei derived from embryonic blastomeres or larval intestine, respectively, would support the development of fertile adults after transplantation to enucleated oocytes [1, 2], suggesting that these nuclei had not been irreversibly modified during differentiation. On the other hand, nuclei derived from adult frogs were never able to support full development, suggesting an irreversible loss of totipotency [3].

Initial studies in mammals argued for an even more limited developmental potential, with the inner cell mass of the blastocyst and its cultured derivatives being the most advanced stages of development that could be achieved [4, 5]. Recent studies have indicated, however, that nuclei derived from cultured cells obtained from mammary tissue of adult ewes [6] and from ovarian granulosa cells of mice [7] are able to support full development to term. These dramatic findings have reopened the question of the irreversibility of nuclear differentiation and of the capability of ooplasm to reprogram somatic nuclei to produce totipotent state. Nonetheless, the molecular mechanisms underlying the resetting of the developmental program following the transplantation of nuclei into oocytes remain almost entirely unknown.

To better understand the reprogramming of mammalian nuclei, several approaches have been used to characterize the cellular and molecular changes occurring during the early development of nuclear-

transplant embryos. One approach has been to analyze the expression of stage-specific proteins and antigens. In mice, differentiated embryonic and endoderm-like somatic nuclei obtained from a teratocarcinoma, when transferred to an enucleated 1-cell embryo, are able to direct synthesis of certain proteins known as the transcription-requiring complex that are markers of embryonic genome activation [8]. Others have observed that ooplasm is able to modify the composition of the nuclear lamina of transplanted mouse 8- and 16-cell blastomere nuclei [9]. Moreover, when pig 16-cell blastomeres are fused to enucleated and activated secondary oocytes, small nuclear ribonuclear proteins (snRNP) disappear whereas nuclear lamins A/C reappear, reflecting the pattern found at the 1-cell stage [10, 11]. In cattle, ultrastructural studies indicate that complete reprogramming is observed with respect to the blebbing activity of the nuclear envelope and transcription of heterogeneous nuclear RNA, whereas only partial reprogramming of nucleolar fine structure occurs [12]. Moreover, effects of the cell cycle stage of host ooplasts on the remodeling and developmental competence of reconstructed embryos has been demonstrated previously using both somatic and blastomere donor nuclei [13, 14].

The most obvious morphological change observed in transplanted nuclei is a rapid swelling reported in amphibians [15]and in mammals, including mice [13], rabbits [14, 16] and pigs [17]. Although the precise causes of nuclear swelling are yet unclear, exchange of both acidic and basic proteins between donor nuclei and cytoplasm has been observed [18, 19]. It may be speculated that ooplasmic proteins imported into the nucleus mediate structural rearrangement of the chromatin, which functionally resets it to a totipotent state. Similar events may occur naturally after fertilization, when ooplasmic nucleoplasmin and core histones mediate remodeling of the sperm chromatin to form the male pronucleus [20, 21].

In addition to the core histones, linker histone H1 appears to be

actively involved in the regulation of gene expression during early embryonic development in several species [reviewed by 22]. Oocytes and early embryos of many species lack the somatic form of histone H1, which first becomes detectable on chromatin at about the stage when the embryonic genome becomes transcriptionally active [23-26]. In *Xenopus*, experimental acceleration or delaying of the timing of the switch from embryonic-type to somatic-type H1 correspondingly altered the time when certain mesoderm-inducing genes could be transcribed [27]. These results strongly support a role for changes in histone H1 in the regulation of early embryonic genome activity.

Based on these results, we investigated whether the functional reprogramming of embryonic nuclei that occurs when they are transplanted into ooplasm at different stages after activation is accompanied by changes in their histone H1 complement. We focused our experiments on the bovine system, where embryonic nuclei transplanted into oocytes support development at a much higher frequency than in the mouse.

MATERIALS AND METHODS

Source of oocytes and embryos

Ovaries were collected from a local abattoir, stored in saline at 30-35°C and brought to the laboratory within 2 hours of slaughter. Follicles with diameters between 2 to 8 mm were punctured with a 19-gauge needle and cumulus-oocyte-complexes (COCs) with several layers of cumulus cells and homogeneous oocyte cytoplasm were washed in Hepes-buffered tissue culture medium (TCM-199; Gibco BRL, Burlington, Canada) supplemented with 10% (v/v) fetal calf serum (FCS, Gibco). Groups of 20 COCs were placed in 100 µl of bicarbonate-buffered TCM-199 supplemented with 10% FCS, 50 µg ml^{-1} LH (Ayerst, London, ON,

Canada), 0.5 μg ml^{-1} FSH (Folltropin-V; Vetrepharm, St-Laurent, PQ, Canada), 1 μg ml^{-1} estradiol 17-β (Sigma, St-Louis, MO, USA), 22 μg ml^{-1} pyruvate (Sigma) and 50 μg ml^{-1} gentamicin (Sigma). After 24 hours of maturation in vitro (IVM), oocytes were in vitro fertilized (IVF) using standard protocols [28]. Briefly, COCs were placed in 50 μl drops of Tyrode's medium, supplemented with 0.6% bovine serum albumin (BSA Fraction V, Sigma), lactate, pyruvate, gentamicin and 10 μg ml^{-1} of heparin. Frozen-thawed spermatozoa were washed and centrifuged through a Percoll gradient and diluted at 10^6 live spermatozoa ml^{-1}. At 20 hours post-insemination, COCs were denuded of cumulus cells by brief shaking and the presumed fertilized zygotes were transferred to 50 μl drops of Menezo B2 medium (MB2; Pharmascience, Paris, France) supplemented with 10% FCS in the presence of bovine oviductal epithelial cells (BOEC). All cultures were performed in drops under equilibrated mineral oil at 39°C in a humidified atmosphere of 5% CO_2 in air.

Nuclear transplantation protocols

Several nuclear transplantation protocols were compared to assess the ability of oocytes at different cell cycle stages to modify the chromatin of morula-stage nuclei (Fig. 1). In the first protocol (Group 1), oocytes were denuded of cumulus cells after 24 hours of IVM, placed in PBS containing 7.5 μg ml^{-1} cytochalasin B (Sigma), and approximately 30% of the cytoplasm adjacent to the first polar body was removed. After microsurgery, oocytes were placed in medium containing 5 μg ml^{-1} Hoechst 33342 for 15 min and exposed briefly to ultraviolet irradiation to verify by the absence of chromatin that enucleation was complete. A single blastomere derived from an in vitro-produced morula at day 5 after IVF was introduced into the perivitelline space of the enucleated oocyte and the resulting couplet was placed in a 0.3 M mannitol solution containing 0.1 mM $MgSO_4$ and 0.05 mM $CaCl_2$ and exposed to a 1.5 KV

electric pulse lasting 70 μsec. Previous experiments have indicated that exposure to an electric pulse at 26 hours causes low rates of activation. After electrical stimulation, oocytes were washed in TCM-199 and 1 hour later were exposed to 5 μM ionomycin (Sigma) to induce parthenogenetic activation, washed and placed in MB2 with BOEC for culture. For Group 2, oocytes were matured for 24 h, enucleated, exposed to ionomycin at 28 hours and a morula nucleus was introduced 1 hour later by fusion as described for Group 1.

For Groups 3 and 4, oocytes were denuded of cumulus cells at 30 hours after IVM, exposed to an activation treatment with ionomycin and returned to the incubator for 2 hours to allow for extrusion of the second polar body. During or immediately after polar body extrusion, telophase II-stage oocytes were exposed for 15 min to medium containing cytochalasin B and approximately one-tenth of the cytoplasm adjacent to the second polar body was removed [29]. A morula-stage blastomere was introduced either immediately (Group 3) or 4 hours later (Group 4) into the perivitelline space and the resulting couplet was exposed to an electric pulse to induce fusion, as described above. Although these cells contained no host chromosomes, we refer to them as interphase to indicate that they were used for fusion several hours after oocyte activation.

In the third protocol (aged metaphase enucleation; Group 5), oocytes were matured for 24 h, the chromosomes were removed, and the oocytes were returned to the IVM drops for another 18 h. The extended maturation protocol (aging) is used routinely in nuclear transplantation procedures to improve the developmental competence of reconstructed embryos [30]. After aging (i.e., 42 hours of IVM), the ooplasts were cooled to 12°C for 3 hours and then fused to a morula-stage blastomere.

Drugs

Stock solutions of drugs were prepared in dimethyl sulfoxide

(DMSO) at concentrations of 10 mg ml^{-1}, and working solutions of α - amanitin (100 μg ml^{-1}; Boehringer), aphidicolin (1.5 μM; Boehringer), cycloheximide (10 μg ml^{-1}, Sigma) and 6-dymethylaminopurine (6-DMAP; 3 mM; Sigma) were prepared by appropriate dilution of stock into MB2 medium supplemented with 10% FCS. Nuclear-transplant embryos were exposed to these drugs as described in the Results.

Synchronization of blastomeres

Morula-stage embryos at day-5 after IVF were placed into 0.33 μM nocodazole in MB2 culture medium for 12 hours to synchronize cells at metaphase. After nocodazole treatment, embryos were exposed to a 0.1% pronase solution for 3 minutes to remove the zona pellucida followed by disaggregation using a fine bore pipette. Separated blastomeres were placed individually into 10 μl drops and observed hourly to verify the time of cleavage. Cleaved blastomeres were removed (defined as at G1-phase) and fused to interphase ooplasts (Group 3). Following fusion, the nuclear-transplant embryos were incubated in the presence of 6-DMAP, cycloheximide, or medium alone. In general, blastomeres were fused to oocytes within 1.5 to 2 hours after they had cleaved.

Immunocytochemistry

Groups of nuclear-transplant oocytes and embryos at 1, 3, 6, 9, 12, 16, 24, 48, 96 and 168 hours after fusion were fixed in 10% formalin (Sigma) for 20 min, washed and stored at 4°C in 0.9% saline containing 0.1% Tween-20. To detect somatic histone H1, fixed oocytes and embryos were incubated in a blocking solution (PBS, 3% BSA, 0.5% Triton X-100) for 1 hour at room temperature, then transferred to anti-histone H1 antibody (raised in rabbit using histone H1 from rat thymus and affinity-purified [25, 31, 32]diluted in 1:50 in blocking solution, and incubated overnight at 4°C. This antibody has previously been shown to recognize somatic H1 subtypes but not H1 subtypes present in mouse and bovine

oocytes and early embryos [25, 26, 33]. The cells were then washed twice in blocking solution, incubated in fluorescein-conjugated goat anti-(rabbit IgG) diluted 1:100 in blocking solution for 1 hour at room temperature, and washed as above. Specimens were mounted on slides in a mounting medium containing Moviol (Hoechst, Montreal), the DNA stain DAPI (1 μg ml^{-1}), and the anti-fading agent, DABCO. They were examined using standard epi-fluorescence optics. Nuclear diameters were measured using an ocular micrometer.

Statistical analysis

Analysis of nuclear diameters in nuclear-transplant embryos was performed by analysis of variance using the Tukey-Krammer HSD test, with the significance level set at 5%. Frequencies of somatic H1 staining among groups of nuclear-transplant embryos were analyzed by chi-square.

RESULTS

Loss of immunoreactive histone H1 from morula nuclei following transplantation into oocytes

The ability of nuclei obtained from blastomeres or differentiated somatic cells, when transplanted into activated oocytes, to direct embryonic development is considered to indicate that these nuclei have been functionally reprogrammed by the oocyte cytoplasm. To investigate whether this presumed reprogramming is accompanied by changes to histone H1 on chromatin, individual blastomeres derived from morula-stage embryos were fused to oocytes whose chromosomes had been removed (ooplasts). Depending on the experiment, the recipient ooplast was parthenogenetically activated at different times before or after introduction of the morula nucleus (Fig. 1). At different times following

fusion, the nuclear-transplant embryos were fixed, reacted with an antibody recognizing somatic H1, and examined using immunofluorescence microscopy.

We observed that the behavior of the donor nucleus depended on whether it was introduced near the time of oocyte activation or several hours later. When introduced 1 hour before or 1 hour after activation of the host oocytes, when the oocytes were near metaphase (Groups 1 and 2), the morula nucleus remained in interphase in about half of the recipients. In the other half of the recipients, the nucleus underwent chromosome condensation within the oocyte cytoplasm. This condensation was observed regardless of the time post-fusion when the cells were fixed. In many cases, the cells contained fragmented or apparently pycnotic chromatin derived from the blastomere nucleus (data not shown). In contrast, when the nucleus was introduced 2.5 hours or 6 hours after activation, when the oocytes were in interphase (Groups 3 and 4), the morula nucleus remained in interphase in every case. It is possible that high metaphase-promoting factor (MPF) activity in the oocytes used for fusion near the time of activation promoted condensation of the donor chromatin, whereas low MPF activity in the oocytes used several hours after activation allowed the donor nuclei to remain at interphase [29].

To examine the fate of the somatic H1 associated with the morula-stage chromatin during residence in the ooplasm, the nuclear-transplant embryos stained using the anti-somatic H1 antibody were examined using epi-fluorescence. In the cases where the donor chromatin condensed in the ooplasm, no staining was observed in any case (data not shown). When the donor nucleus remained at interphase, somatic H1 staining was prominent on the donor nuclei during the first several hours after transplantation into oocytes (Fig. 2). In oocytes fixed at later times, however, the intensity of the signal declined and it ultimately became undetectable. Thus, incubation of morula nuclei in the cytoplasm of activated oocytes led to a loss of immunoreactive somatic H1 from the

nuclei.

The kinetics of loss of immunoreactive somatic H1 from the morula nuclei differed, however, depending on the cell cycle stage of the host cytoplasm (Table 1). When the host oocytes were near metaphase at the time of fusion (activated 1 hour before or 1 hour after fusion), somatic H1 became undetectable in the donor nucleus after between 3 and 6 hours of residence in the ooplasm. However, when the hosts were at early interphase at the time of fusion (activated 2.5 hours before fusion), loss of somatic H1 occurred between 6 and 9 hours after fusion, and when they were fused later in interphase (activated 6 hours before fusion) somatic H1 persisted until 12 to 16 hours post-fusion. These results indicate that the loss of immunoreactive somatic H1 from the morula nuclei occurred more rapidly in oocytes that were near metaphase at the time of fusion than in oocytes that were in early or later interphase.

It may be seen in Fig. 2 that the morula nuclei increased in volume during residence in the ooplasm. This raised the possibility that the loss of immunoreactive histone H1 might be linked to nuclear swelling and, if swelling occurred more slowly in the interphase recipients, this could account for the relatively slow loss of immunoreactive H1 in these ooplasts. To investigate this, we measured the diameter of the nucleus of each embryo at the different times after nuclear transplantation. In some cases, two nuclei were present in a single ooplast and these were excluded from the calculation. The results are shown in Fig. 3, together with the frequency of histone H1 immunoreactivity, which has been obtained from Table 1 and plotted as a histogram. It may be seen that, at 6 hours after fusion when somatic H1 was not detectable in the metaphase recipients yet remained in the interphase recipients, there was no significant difference in mean nuclear diameter between these groups. Similarly, at 9 hours after fusion when somatic H1 remained detectable in the 6-hr interphase recipients, the mean diameter of these nuclei did not differ from those in the metaphase recipients. By 16 hours after fusion

when somatic H1 was not detectable in any group, the mean diameter of the transplanted nuclei was similar in the metaphase and two interphase groups. Thus, the morula nuclei did not enlarge more slowly in the interphase environment than in the metaphase environment, indicating that differential nuclear growth cannot underly the relatively slow loss of immunoreactive H1 observed in interphase recipients. The results also indicate that, although a general correlation exists between nuclear growth and loss of immunoreactive H1, there appears to be no direct connection between these two processes.

When aged oocytes were used as recipients, the loss of immunoreactive H1 also occurred relatively slowly, becoming undetectable between 12 and 16 hours post-fusion (Table 1). As nuclear swelling also was retarded or inhibited in these oocytes (Fig. 3), it may be that aged cytoplasm differs in several respects from younger cytoplasm, meaning that these results cannot be easily compared with those obtained using metaphase and interphase recipients. The results may suggest, however, that loss of H1 immunoreactivity depends on factors whose activity declines during aging in vitro of inactivated oocytes.

Role of cellular synthetic processes in the loss of immunoreactive histone H1 from morula chromatin

To study molecular mechanisms underlying the loss of immunoreactive somatic histone H1 from the chromatin of donor nuclei during residence in activated oocyte cytoplasm, embryos were constructed using telophase-enucleated ooplasts at 2.5 hours after activation and then were cultured for 12 hours in the presence of an inhibitor of DNA replication (aphidicolin) or transcription (α -amanitin), or for 12, 16 or 20 hours in the presence of an inhibitor of protein synthesis (cycloheximide) or protein phosphorylation (6-DMAP), before being fixed and processed for immunofluorescence. The 12-hour incubation period was chosen because immunoreactive H1 has been lost from morula nuclei in 2.5-hour

telophase-enucleated recipient ooplasts by this time (Table 1).

Nuclear diameter was significantly reduced in nuclear-transplant embryos exposed to aphidicolin (25.8 vs. 39.4 μm, $P \leq 0.01$), suggesting that the extent of nuclear swelling was partially dependent on DNA replication. In contrast, α -amanitin had no detectable effect on nuclear diameter (37.9 μm). In nuclear-transplant embryos exposed to either drug, no immunoreactive somatic H1 was detected at 12 hours after fusion (aphidicolin: 0/16; α -amanitin: 0/14). These results suggest that loss of immunoreactive somatic H1 occurred independently of DNA replication and transcription.

When the nuclear-transplant embryos were incubated in either cycloheximide or 6-DMAP, nuclear swelling was reduced at 12 hours post-fusion compared to controls (Fig. 4). During the next 8 hours of incubation, the nuclei of the cycloheximide-treated embryos enlarged to become similar in size to controls, whereas the nuclei of 6-DMAP-treated embryos remained relatively small. In the presence of either drug, immunoreactive histone H1 remained detectable in a substantial portion of the donor nuclei after 12 hours of residence in ooplasm (Table 2), even though it was undetectable in all control cells processed at the same time. At 16 or 20 hours post-fusion, however, somatic H1 was no longer detectable in the majority of the cases. These results suggest that the loss of immunoreactive somatic H1 was delayed in the absence of coincident protein synthesis or protein phosphorylation. This effect was transient, however, and variable in that it was manifested only in a proportion of the cases.

To test whether the variable effects of cycloheximide and 6-DMAP on the loss of immunoreactive somatic H1 were linked to cell cycle asynchrony of the donor morula nuclei, the following experiment was conducted. Embryonic cells were synchronized by using nocodazole to arrest each blastomere when it reached metaphase, then transferred into nocodazole-free medium. One hour after cleavage, newborn blastomeres

whose nuclei were expected to be at G1 were fused to host oocytes. These nuclear-transplant embryos were cultured in the presence of cycloheximide or 6-DMAP, and then fixed. Non-synchronized cell nuclei were used in parallel and these results are included in Table 2. It may be seen that, using the G1 nuclei, immunoreactive somatic H1 was no longer detectable by 12 hours in most cases despite the presence of cycloheximide or 6-DMAP (Table 2). By contrast, using non-synchronized blastomeres, immunoreactive H1 remained present at 12 hours post-fusion in between one-third and one-half of the embryos. These results suggest that loss of immunoreactive somatic histone H1 from G1 nuclei, as compared to from non-synchronized nuclei, was less dependent on coincident protein synthesis and phosphorylation.

Re-appearance of immunoreactive somatic histone H1 on the chromatin of nuclear-transplant embryos

During development in vitro of normal bovine embryos, immunoreactive histone H1 first becomes detectable at the 8- to 16-cell stage [26]. We wished to examine whether the nuclear-transplant embryos would exhibit the same developmental regulation (Table 3, Fig. 2). No immunoreactive H1 could be detected either 2-cell or 4-cell embryos that developed from the nuclear-transplant oocytes. At the 8- to 16-cell stage, however, most blastomere nuclei were immunoreactive. At the blastocyst stage, the nuclei of both the inner cell mass and trophectodermal cells stained positively for somatic H1. No difference in the timing of the re-appearance of immunoreactive H1 on the chromatin was apparent among the different experimental groups (Table 3). These results indicate that, in nuclear-transplant embryos, immunoreactive somatic H1 becomes detectable on the chromatin at the same stage as in unmanipulated embryos developing in vitro.

DISCUSSION

To better understand the chromatin structural modifications that may accompany functional reprogramming of foreign nuclei that have been transplanted into mammalian oocytes, we have assessed changes in the histone H1 complement of bovine morula nuclei fused to ooplasts. We show that somatic histone H1 becomes immunologically undetectable during the first cell cycle of the nuclear-transplant embryos and subsequently becomes newly detectable on the embryonic chromatin during the fourth cell cycle. Moreover, the kinetics of histone H1 disassembly is clearly affected by the cell cycle stage of the recipient cytoplasm. Finally, we show that the molecular mechanism implicated in the disassembly of somatic histone H1 in reconstructed oocytes is influenced by newly synthesized proteins and by protein kinase activity. These findings identify a specific molecular change in somatic chromatin exposed to oocyte cytoplasm and may provide insight into the mechanisms by which the ooplasm reprogrammes differentiated nuclei following nuclear transplantation so that they acquire developmental totipotency.

Loss and re-appearance of immunoreactive somatic histone H1 after nuclear transplantation

Using an antibody that recognizes the somatic subtypes of histone H1, we observed that immunoreactive H1 disappears from the chromatin of morula-derived blastomere nuclei after transplantation into ooplasts. Thus the ooplasm modifies the morula chromatin in some manner. This modification could include the removal of somatic H1 from the chromatin or alternatively might alter the chromatin such that somatic H1 is masked from the antibody. We favour the first alternative for two reasons. First, in the mouse, the somatic H1 subtypes are not detectable in oocytes by immunoblotting [25]. Unfortunately thousands of mouse oocytes were

required in the latter experiment, an unrealistic option to confirm the loss of somatic H1 from nuclear-transplant embryos. Second, in the amphibian, *Xenopus laevis*, an extract of egg cytoplasm remodels somatic nuclei by removing the somatic forms of histone H1 [34]. In this case, the somatic H1 is replaced by the oocyte-specific histone H1 variant, B4. Interestingly, somatic H1 is removed from nuclei even in extracts depleted of histone B4, suggesting this nucleo-protein exchange is not determined by competitive binding of the two proteins [34]. In the experiments reported here, it may be speculated that somatic H1 of the bovine morula nuclei is replaced by a oocyte-specific H1 variant that does not react with the antibody. Although the nature of this putative H1 variant is unknown, mouse oocyte nuclei can be stained using an antibody raised against histone $H1^0$ [33].

In addition to the loss of immunoreactive histone H1, the morula nuclei enlarged considerably in the ooplasm. Between 1 and 16 hours after fusion the mean diameter increased approximately 2.5-fold, representing about a 15-fold increase in volume. Early studies in amphibians demonstrated that somatic nuclei transplanted into fertilized egg cytoplasm decondense and increase in volume similarly to pronuclei [35]and this was later shown to be correlated with the import of cytoplasmic proteins into the nuclei [18]. Exchange of nuclear and cytoplasmic non-histone proteins has also been demonstrated after transplantation of somatic nuclei into enucleated oocytes [19, 36]. A similar nuclear swelling has been observed following transplantation in many mammalian species [13, 14, 17]. Taken together, these results suggest that numerous modifications to the protein complement of chromatin, as exemplified by the changes in histone H1 reported in this study, may occur when somatic nuclei are exposed to oocyte cytoplasm.

When the nuclear-transplant embryos were incubated to permit further development, immunoreactive H1 re-appeared at the 8- to 16-cell stage, which is the stage when it first becomes detectable in

unmanipulated bovine embryos [26]. This time corresponds to the major activation of embryonic gene expression. Indeed, a close temporal relationship exists in several species between the transition to the somatic form of histone H1 and the initiation of major transcriptional activity. In the mouse, where the major transcriptional activation occurs at the 2-cell stage [37], somatic H1 becomes detectable at the 2- or 4-cell stage [25]. In *Xenopus* embryos, somatic H1 begins to accumulate and B4 declines near the mid-blastula transition when there is a major increase in transcriptional activity [38]. This developmentally regulated change in *Xenopus* histone H1 subtype plays an important role in regulating the pattern of activity of specific genes [27]. The fact that the re-appearance of immunoreactive H1 in nuclear-transplant embryos precisely matches that of unmanipulated embryos implies that this chromatin modification serves a specific function during early mammalian embryogenesis as well.

Effects of the cell cycle stage on loss of immunoreactive histone H1

We found that the immunoreactive histone H1 was lost from the morula chromatin more rapidly when the host ooplasts were near metaphase than when they were near telophase or further in the first embryonic cell cycle. This suggests that the cytoplasmic activities that remove immunoreactive histone H1 from the morula chromatin may decline in efficiency as the cell progresses into the first embryonic cell cycle. Several previous studies have suggested that re-programming of somatic nuclei in oocytes may occur more efficiently in metaphase cytoplasm than in interphase cytoplasm. When thymocyte nuclei were transplanted to murine oocytes, they developed into pronuclear-like structures when the hosts were obtained at metaphase but retained their somatic-like nuclear morphology when the hosts were at interphase [13, 39]. In cattle, metaphase ooplasm extinguished transcriptional activity of morula nuclei more efficiently than interphase cytoplasm [40]and, in the rabbit, host ooplasts at the metaphase stage allowed for more extensive

nuclear swelling and better development of nuclear-transplant embryos [14]. Most recently, [7], using cumulus granulosa cell nuclei to clone mice, found that development was improved by prolonged exposure of donor nuclei to metaphase stage ooplasm before activation, although this was not compared with telophase or interphase cytoplasm. Considering these results together with our observation that immunoreactive histone H1 is lost more rapidly in metaphase cytoplasm than in interphase cytoplasm, it may be proposed that metaphase cytoplasm more rapidly molecularly remodels foreign nuclei and this enhances their developmental competence.

Molecular mechanisms controlling loss of immunoreactive histone H1

Neither aphidicolin nor α-amanitin detectably influenced the timing of somatic histone H1 disappearance, implying that the controlling mechanisms do not depend on DNA replication or transcription. This contrasts with the re-appearance of somatic histone H1 on chromatin during early embryogenesis, which requires both DNA synthesis and transcription [25, 26]. Our results suggest that, if the immunoreactive histone H1 is replaced by an oocyte-specific H1 variant, this variant must be present in the ooplasm before the first round of DNA replication or is synthesized through a replication-independent mechanism. In this connection, it was observed [41] that in activated eggs certain histone H1 subtypes are synthesized only at the late 1-cell stage, implying that if histone H1 is assembled into nascent chromatin during the first embryonic S-phase, it is present in the oocyte cytoplasm before fertilization.

Inhibitors of protein synthesis or kinase activities delayed the loss of immunoreactive histone H1 from the morula chromatin in a large proportion of the reconstructed oocytes. Although the identity of protein kinases that may participate in this process is entirely speculative, metaphase-arrested oocytes, which effect a rapid loss of immunoreactive

H1, have high M-phase promoting factor (MPF) activity. It has previously been shown that thymocyte nuclear membranes break down under the influence of residual MPF in newly activated oocytes, although a new nuclear membrane subsequently forms as the cell cycle progresses [39]. Furthermore, in amphibian egg extracts, permeabilization of the nuclear envelope of interphase nuclei by addition of chemical reagents or MPF allowed access to the chromatin of cytoplasmic factors capable of modifying chromatin activity [42, 43]. Thus, the faster removal of somatic histone H1 from morula nuclei exposed to ooplasts at metaphase or immediately after activation might be due to MPF activity on the nuclear membrane, allowing rapid accessibility of ooplasmic factors to the donor chromatin or a faster exit of immunoreactive histone H1 through the nuclear membrane.

Additionally, metaphase-related kinases could affect the phosphorylation pattern of proteins that mediate the loss of immunoreactive histone H1. Hyperphosphorylated nucleoplasmin removes basic proteins from Xenopus sperm more rapidly than the hypophosphorylated form [21, 34, 44]. Remodeling of the sperm nucleus into the paternal pronucleus is also associated with phosphorylation of core histones [45]. Additionally, phosphorylation of histone H1 can affect its stability within the chromatin [46], suggesting that kinases active in the ooplasm close to the time of activation may affect histone H1 interaction with chromatin by changing its phosphorylation state. These studies suggest that the effect of ooplast cell cycle on the kinetics of somatic H1 disassembly may in part reflect differential activities of protein kinases.

Although both protein synthesis and kinase inhibitors delayed the timing of H1 disassembly in a proportion of the nuclei when non-synchronized cells were used, they had little apparent effect when nuclei were obtained from cells shortly after release from a metaphase block. We infer that these synchronized nuclei were at G1 and that, in the non-

synchronized cells, the nuclei from which loss of immunoreactive histone H1 was not delayed by cycloheximide or 6-DMAP were also at G1. This suggests that on-going protein synthesis and phosphorylation were required for the timely removal of immunoreactive histone H1 from S-phase or G2 nuclei, but not from G1 nuclei.

As the cell fusion technique that we used introduced the morula cell cytoplasm as well as the nucleus, it is possible that donor cells in G1 contain remnants of metaphasic kinase activities that, as discussed above, could promote the removal of histone H1 from the chromatin. Alternatively, the chromatin structure of G1 nuclei may differ from S-phase and G2 nuclei. Phosphorylation of H1 subtypes is minimal at G1 and increases thereafter until mitosis, when it is maximal [47]. These cell cycle-related H1 histone modification changing levels imply that H1 modulates chromatin condensation and decondensation, possibly affecting their stability in chromatin. If these modifications have not been fully reversed in the G1 nuclei, as compared to the S or G2 nuclei, this could explain their different responses to the cycloheximide and 6-DMAP. An additional possibility is that histone H1 is more tightly associated with replicated chromatin than with unreplicated chromatin. Although the difference between G1 and later-stage nuclei that influences their response to cycloheximide and 6-DMAP remain speculative, it is interesting to note that a similar comparison of donor nuclei at different stages of the cell cycle revealed that nuclei obtained at G1 were better able to support embryonic development to the blastocyst stage than were nuclei obtained at later stages [48]. These results are consistent with the view that more rapid molecular alteration of foreign chromatin in ooplasm is correlated with enhanced developmental potential.

Together, these findings identify a specific modification of morula-stage chromatin that accompanies its functional reprogramming following transplantation to oocyte cytoplasm. Further characterization of the molecular mechanisms underlying chromatin reprogramming will

enhance our understanding of the role oocyte cytoplasm plays on the restructuring of gametic chromatin during early embryogenesis. Moreover, knowledge of the factors involved could enhance our ability to completely reverse nuclei from terminally differentiated and/or neoplastic cells to totipotency.

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TABLE 1. Time-dependent loss of immunoreactive somatic H1 in morula-stage nuclei transplanted into ooplasts at different stages of the cell cycle

Time fixed (h post-fusion)	Cell cycle stage of recipient ooplast at time of fusion											
	Metaphase			Early interphase (2.5 h)			Mid-interphase (6 h)			Aged metaphase		
	n	+	-	n	+	-	n	+	-	n	+	-
1	12	11	1	8	8	0	8	8	0	18	17	1
3	17	8	9	4	3	1	11	11	0	10	8	2
6	14	3	11	6	3	3	8	7	1	6	5	1
9	14	0	14	5	1	4	9	9	0	8	6	2
12	18	0	18	10	0	10	11	4	7	9	3	6
16	8	0	8	6	0	6	8	1	7	8	1	7

Note. n represents the number of nuclear-transplant oocytes analyzed for somatic histone H1, (+) the positive stained, and (-) the negative stained.

TABLE 2. Delayed loss of immunoreactive histone H1 in the presence of inhibitors of protein synthesis and phosphorylation

Cell cycle stage of donor nuclei	Time fixed (h post-fusion)	Cycloheximide			6-DMAP		
		n	+	-	n	+	-
Non-synchronized	12	20	8	12	19	11	8
	16	24	2	22	23	2	21
	20	28	4	24	23	5	18
G1	12	22	1	21	17	3	14
	16	15	0	15	19	1	18
	20	19	0	19	10	2	8

Note. n represents the number of nuclear-transplant oocytes analyzed for somatic histone H1, (+) the positive stained, and (-) the negative stained.

TABLE 3. Re-appearance of immunoreactive somatic H1 during embryonic development of nuclear-transplant embryos reconstructed using ooplasts at different stages of the cell cycle

Embryonic stage	Cell cycle stage of recipient ooplast at time of fusion											
	Metaphase			Early interphase (2.5 h)			Mid-interphase (6 h)			Aged metaphase		
	n	+	-	n	+	-	n	+	-	n	+	-
2-cell	13	0	13	6	0	6	9	0	9	4	0	4
4-cell	11	0	11	7	0	7	8	0	8	5	0	5
8- to 16-cell	8	8	0	6	6	0	9	9	0	8	7	1
Blastocyst	8	8	0	4	4	0	12	12	0	10	10	0

Note. n represents the number of nuclear-transplant oocytes analyzed for somatic histone H1, (+) the positive stained, and (-) the negative stained.

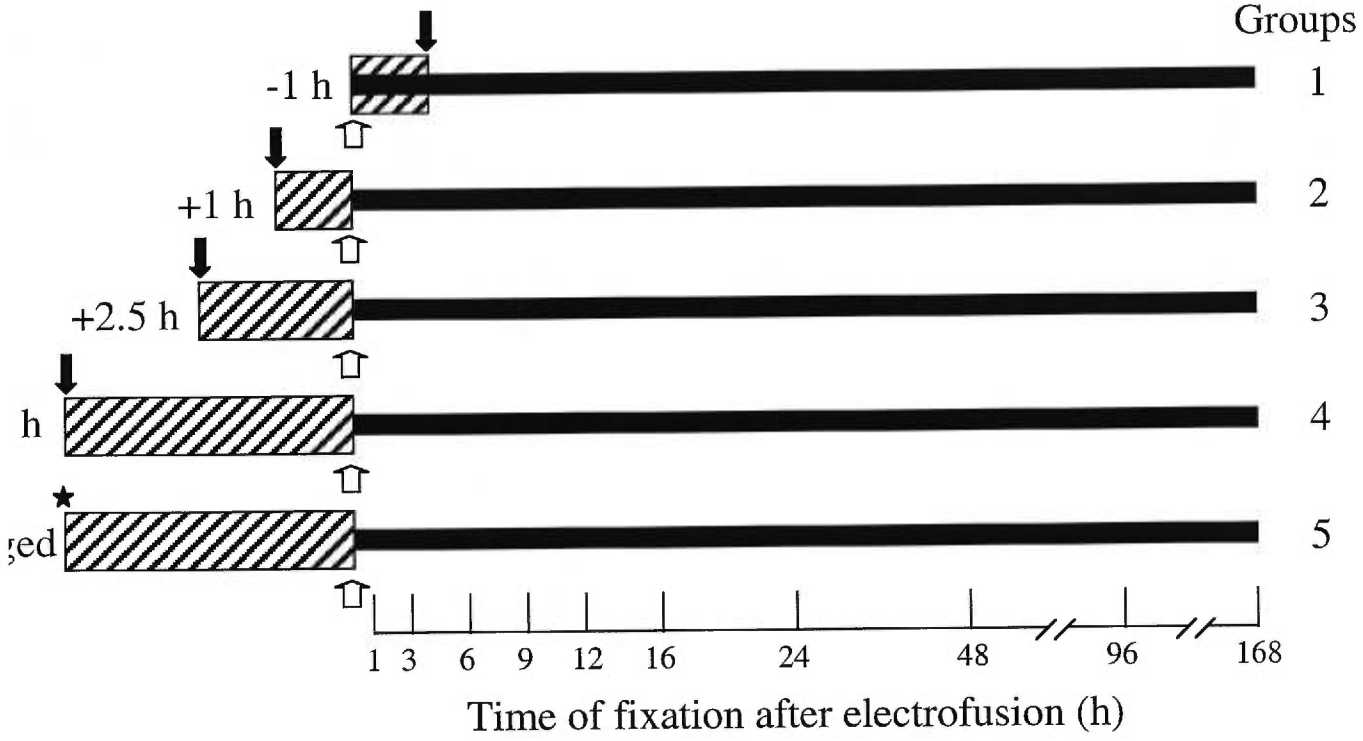
Groups
-1 h
1
+1 h
2
+2.5 h
3
h
4
ged
5
1 3 6 9 12 16 24 48 96 168
Time of fixation after electrofusion (h)

Figure 1.

Schematic representation of the experimental protocol used to reconstruct bovine oocytes. Donor nuclei derived from morula-stage blastomeres were fused (white arrows) at different times (hatched box) in relation to oocyte activation (black arrow), i.e. fusion at 1 hour before (Group 1), at 1 hour (Group 2), 2.5 hours (Group 3), 6 hours after activation (Group 4), and using aged host oocytes (Group 5; black star represents time of exposure to low temperature). Reconstructed oocytes and embryos were fixed for immunocytochemical detection of somatic histone H1 at different times at the 1-cell (1, 3, 6, 9, 12 and 16 hours), 2-cell (24 hours), 4- to 8-cell (48 hours), 8- to 16-cell (96 hours) and blastocyst (168 hours) stages.

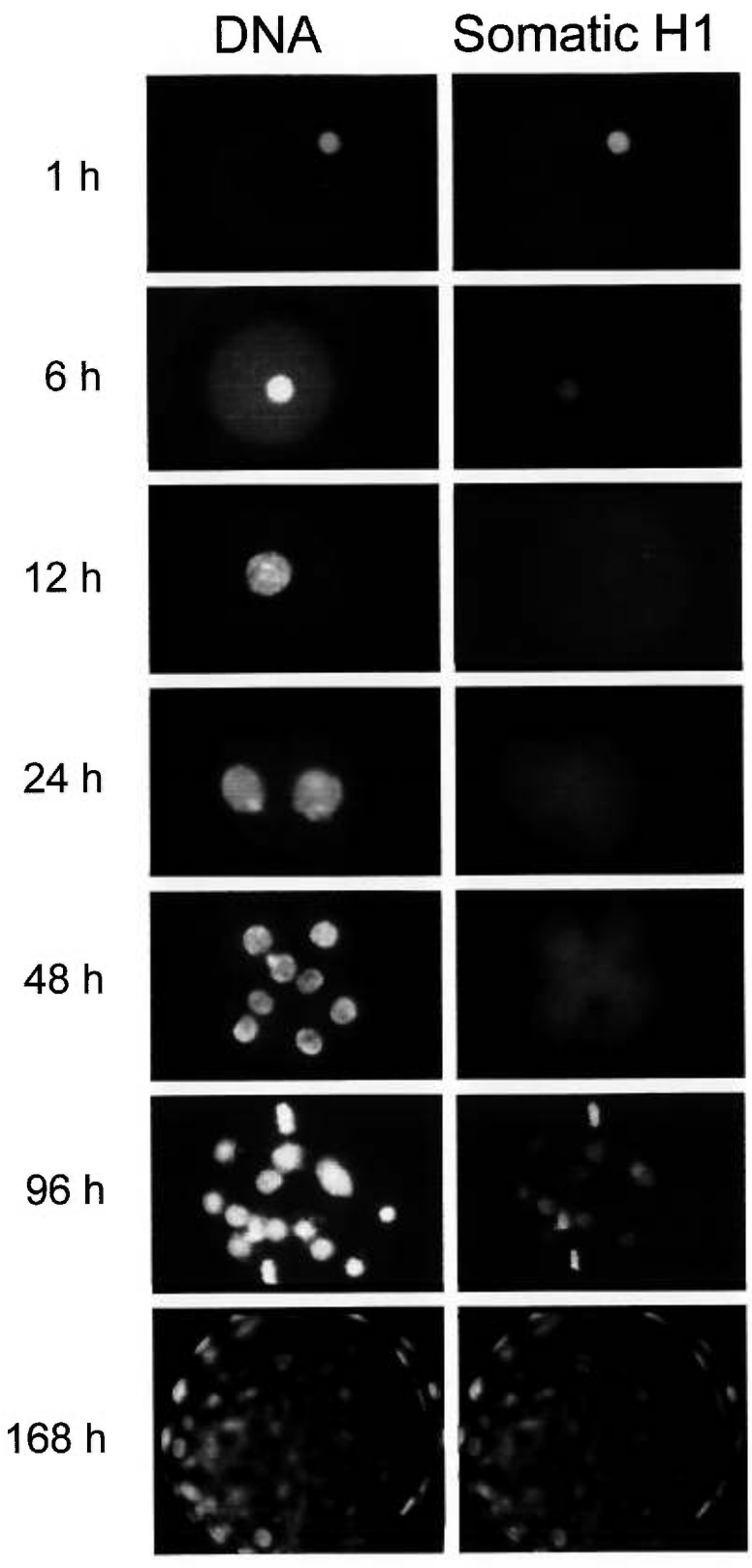
DNA
Somatic H1
1 h
6 h
12 h
24 h
48 h
96 h
168 h

Figure 2.

Epi-fluorescence staining of reconstructed bovine embryos showing the disassembly and re-assembly of somatic histone H1 onto chromatin at different times after nuclear transplantation. Representative examples of nuclear-transplant embryos obtained by fusion of morula-stage blastomeres to host cytoplasts at 2.5 hours after activation are shown after DNA staining with DAPI (left panel) and immunostaining (FITC) for somatic histone H1 (right panel). 400 X magnification.

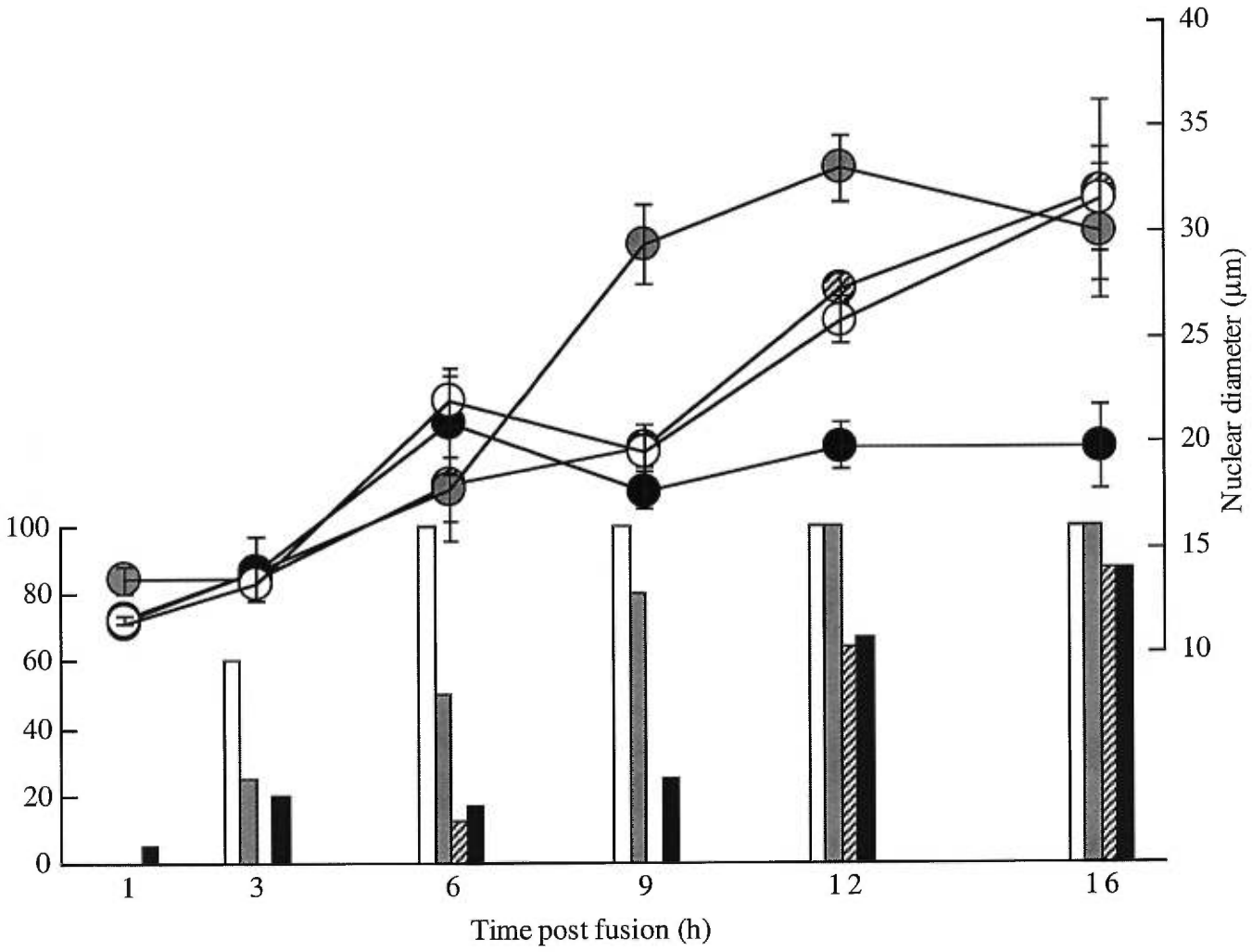
100
80
60
40
20
0
1
3
6
9
12
16
Time post fusion (h)
40
35
30
25
20
15
10
Nuclear diameter (µm)

Figure 3.

Changes in the diameter of donor nuclei at different times after nuclear transplantation (upper graph) and relationship to the loss of H1 immunoreactivity (lower graph). Means and standard errors (vertical bars) of nuclear diameters between 1 and 16 hours after fusion were obtained from reconstructing embryos using host ooplasts in metaphase (white), after activation at 2.5 hours (gray), 6 hours (hatched) and using aged ooplasts (black).

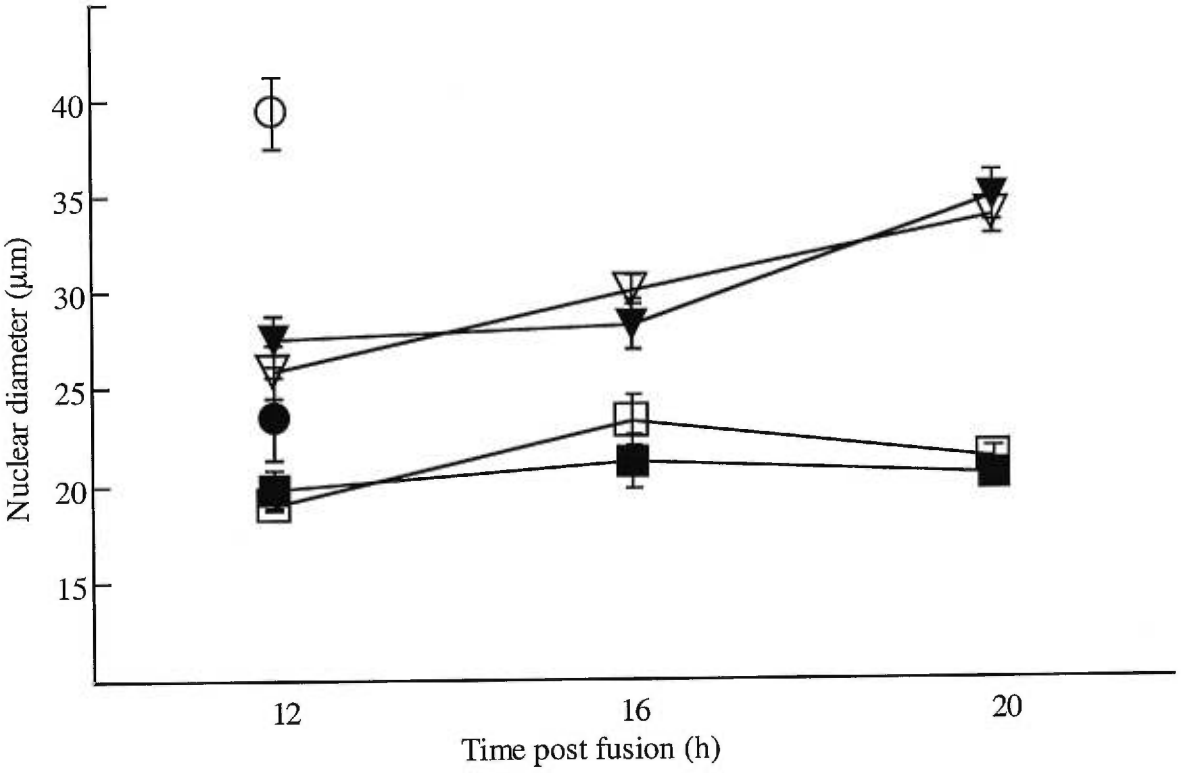
Nuclear diameter (μm)
40
35
30
25
20
15
12
16
20
Time post fusion (h)

Figure 4.

Effect of inhibitory agents on the remodeling of donor nuclei after transplantation to host cytoplasts at 2.5 hours post activation. Donor morula-stage nuclei were either non-synchronized (blank) or in G1-phase (black) at the time of nuclear transplantation. After electrofusion, embryos were cultured for 20 hours in the presence of 10 μg ml^{-1} cycloheximide (triangles), 3 mM 6-dymethylaminopurine (squares) or in B2 medium alone as controls (circles). Vertical bars represent standard errors of mean nuclear diameters at different times of culture.

Article 4

(En préparation)

Cell cycle stage of nucleus and host cytoplasm control the loss of immunoreactive somatic histone H1 from mouse blastomere chromatin after nuclear transfer

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Key words: somatic histone H1, cell cycle stage, chromatin remodeling, nuclear transfer, mouse.

Abstract

When nuclei of somatic cells are transplanted into oocyte cytoplasm, they can in some cases support normal development of the recipient from zygote to term. These findings indicate that the host ooplasm in some manner reprograms the gene expression pattern of the donor nuclei. The cell cycle stage of both the host and the recipient are known to influence the reprogramming process. The basis for these cell-cycle effects, however, is unknown. Somatic histone H1 is present in somatic nuclei, but becomes immunologically undetectable after these are transplanted into oocytes. We have investigated whether the altered H1 immunoreactivity, like nuclear reprogramming, is governed by the cell cycle stage of donor and recipient. To obtain donor nuclei at specific stages of the cell cycle, 4-cell stage mouse embryos were cultured in the presence of nocodazole to synchronize the blastomeres at metaphase. The blastomeres were removed from nocodazole and used either immediately (metaphase) or 3 h (G1/early S phase) or 8 h (late S/G2 phase) after cleavage. Host oocytes were at metaphase II or were ethanol-activated and enucleated/fused at 3 h (telophase) or enucleated/fused at 8 h (interphase) post-activation. Reconstructed embryos were fixed at specific times after fusion and processed for immunofluorescent detection of somatic histone H1. Immunoreactivity was lost more quickly from donor nuclei at metaphase than from donor nuclei at G1/early S or late S/G2 stage. This was particularly evident when host cytoplasts were at interphase where, at 4 h post-fusion, somatic H1 was undetectable in most metaphase chromatin whereas it remained present in most G1/early S and all late S/G2 nuclei. In addition, regardless of the stage of the donor nucleus, immunoreactivity was lost most rapidly when the recipient cytoplast was at metaphase and most slowly when the recipient was at interphase. Thus, the cell cycle stage of both the donor nucleus and the host cytoplast influenced the timing of loss of somatic H1

immunoreactivity from donor nucleus. To examine the role of host nuclear components independently of cell cycle stage, activated oocytes were enucleated at 3 h (telophase) or 7 h (interphase) post-activation or were not enucleated. All groups were incubated until 8 h post-activation, and then to these recipients were fused G1/S blastomeres. Somatic H1 immunoreactivity was lost more slowly from the nuclei resident in oocytes that had been enucleated at interphase or not enucleated than from those that had been enucleated at telophase. Thus, the presence of the host nucleus delayed the loss of H1 immunoreactivity from the donor nucleus, suggesting that the host nucleus may sequester factor(s) involved in the loss of somatic H1 immunoreactivity. Taken together with the evidence that the cell cycle stage of donor nucleus and host cytoplast as well as host nuclear components influence the activity of the transferred nucleus, the results support the view that modifications to the histone H1 complement may underlie or constitute a component of the developmental reprogramming of somatic nuclei in oocyte cytoplasm.

Introduction

Elucidating the molecular mechanisms involved in the resetting of the developmental program after the transplantation of nuclei into host oocytes is an intriguing biological question, and represents a decisive step for the application of nuclear transfer technologies in biomedicine and biotechnology. Although several recent studies have shown that nuclei derived from differentiated cells from adult mammals can be completely reprogrammed to support development to term (Kato et al. 1998; Wakayama et al. 1998), the molecular modifications accompanying the reprogramming of the transplanted nuclei remain almost entirely unknown. Initial studies in amphibians performed to investigate how nuclei are remodeled after transplantation into egg cytoplasm identified

several morphological and functional modifications. The changes observed include nuclear decondensation, induction of DNA synthesis in non-dividing quiescent cells, influx of cytoplasmic proteins into the nuclei, bi-directional exchange of proteins between nuclei and cytoplasm, cessation of RNA synthesis by the transplanted nuclei, and repression and reactivation at the normal stage of embryonic development of specific genes (review by Gurdon, 1986).

Mammalian somatic-cell nuclei transplanted into oocyte cytoplasts also undergo several modifications that are suggestive of reprogramming, including nuclear swelling (Czolowska et al. 1984) and changes in the patterns of nuclear functions such as RNA (Dyban et al. 1988) and protein synthesis (Howlett et al. 1987; Latham et al. 1991). Moreover, differentiated embryonic and endoderm-like somatic nuclei transferred to enucleated 1-cell embryos are able to direct synthesis of a set of proteins known as the transcription-requiring complex that are markers of embryonic genome activation (Latham et al. 1991). Although the above-cited studies indicate that oocyte cytoplasts are able to reprogramme nuclei, others have identified abnormal patterns of nuclear activity in reconstructed one-cell stage embryos. A quantitative study of the pattern of protein synthesis performed in mice revealed significant alterations in the synthesis of several proteins, implying that 8-cell stage nuclei are unable to recapitulate completely the normal progression of changes in the pattern of protein synthesis that occur during the 2-cell stage (Latham et al. 1994). Moreover, early 1-cell embryos were unable to restore the activity of repressed promoters after the transfer of 2 cell nuclei into oocytes (Henery et al. 1995). Overall, notwithstanding the progress in characterizing different functional aspects of transplanted nuclei, the results remain inconsistent and little is known of the modifications occurring to the chromatin itself.

The importance of the cell cycle stage concerning the compatibility between donor nuclei and host cytoplasm at the time of embryonic

reconstruction has been demonstrated in mouse nuclear transplants both within and across cell cycles (Smith et al. 1988). Several other studies in different species have confirmed the influence of the cell cycle on the development potential of nuclei derived from embryonic and somatic cells (Otaegui et al. 1994; Wakayama et al. 1998; Collas et al. 1992; Campbell et al. 1994; Tsunoda, Kato, 1997). Moreover, the remodeling of somatic (Szöllösi et al. 1988) and embryonic (Collas, Robl, 1991) nuclei, as measured by the swelling rate, was more extensive with metaphase than interphase cytoplasts. Chromatin replicative and transcriptional activity of transplanted nuclei was modified faster and more effectively when using metaphase than interphase cytoplasts. Together, these results indicate that the cell cycle stage influences the reprogramming capabilities of reconstructed embryos and, therefore, may act directly on the remodeling of structural components of the chromatin of transplanted nuclei.

It has long been speculated that the developmental modifications occurring to the chromatin during cellular differentiation affect the capability of transplanted nuclei to regain totipotency. This idea is supported by the poor development potential of mouse nuclei derived from more advanced embryonic stages compared with those obtained before or after a few cleavages (McGrath, Solter, 1984). Moreover, as evidenced in other species, nuclei derived from early stage embryos show higher developmental potential (Campbell et al. 1994) than those derived from somatic cells (Campbell et al. 1996; Wilmut et al. 1997). A plausible explanation for this developmental restriction may involve structural modifications to the chromatin during cellular differentiation, which may interfere with a complete remodeling of transplanted nuclei. One of the first differentiation events, which is probably regulated by structural modification of the chromatin, is the transcriptional activation of embryonic genome (Nothias et al. 1995). Adenot et al. (1997) showed that this process is preceded by modifications to the pattern of core

histone acetylation, a structural modification involved in the regulation of chromatin activity (Grunstein, 1997).

Linker histone H1 also appears to be actively involved in the regulation of gene expression during early embryonic development in several species (reviewed by Clarke et al. 1998). In *Xenopus*, oocytes and early stage embryos lack the somatic form of histone H1, which first becomes detectable on chromatin at about the stage when the embryonic genome becomes transcriptionally active (Ohsumi, Katagiri, 1991). Moreover, experimental acceleration or delay of the timing of the switch from embryonic-type to somatic-type H1 correspondingly altered the time when certain mesoderm-inducing genes could be transcribed (Steinbach et al. 1997), supporting the involvement of histone H1 in the regulation of early embryonic genome activity. In mice and cattle, somatic H1 cannot be detected by immunocytochemical means on the chromatin until the time of the major zygotic transcriptional activation in each species (Clarke et al. 1992; Smith et al. 1995), suggesting that linker histones are involved in the early differentiative steps in mammals and supporting their use as a molecular marker to study the remodeling of chromatin structure and function after nuclear transplantation. Therefore, our main objective in the present study was to investigate the role of the cell cycle in the remodeling of the somatic histone H1 component of donor chromatin after nuclear transplantation in mice.

Material and methods

Collection and culture of mouse oocytes and embryos

Hybrid F1 females (C57B1/6 x CH3; Charles River Canada Inc., St.-Constant, Canada), were superovulated by intraperitoneal injection of 5 IU of equine chorionic gonadotrophin (PMSG; Folligon; Ayerst, Montreal, Canada) and 5 IU of human chorionic gonadotrophin (hCG; Ayerst) given

46-48 h apart. Oocytes used as host cytoplasms were collected from the ampullae of the oviducts at 15-18 h after hCG injection. Cumulus cells were removed by treatment with 0.1 % (w/v) hyaluronidase (Sigma, St. Louis, MO) in Hepes buffered CZB medium (Chatot et al. 1989). The oocytes then were placed in 50-µl droplets of glucose-free, bicarbonate-buffered CZB medium under mineral oil (Sigma), at 37 °C in a humidified atmosphere of 5% CO_2 in air until activation or nuclear transfer. Host oocytes were parthenogenetically activated at 20-24 h after hCG injection by exposure to 7 % ethanol (v/v) in Hepes-buffered CZB medium for 5 minutes, washed three times and cultured in bicarbonate-buffered CZB medium to allow the extrusion of the second polar body and pronuclear formation. To produce nuclear-donor embryos, superovulated females were paired with CD-1 males (Charles River) and inspected the following morning for copulation plug. Embryos were flushed from the oviducts at 56-h post hCG and good quality 4-cell stage embryos were selected.

Synchronization of blastomeres

Four-cell embryos were cultured in the presence of 0.33 µM methyl(5-[2-thienylcarbonyl]-1H-benzimidazol-2-yl)carbamate(nocodazole; Sigma) in CZB medium for 6-10 h to synchronize cells at metaphase (Samaké, Smith, 1996). After nocodazole treatment, embryos were used either immediately (metaphase stage) or washed several times and cultured for 3 (G1/early S stage) or 8 h (late S/G2 stage). As the cleavage of arrested blastomeres occurred 0.5-1 h after release from nocodazole, nuclei were thus used at 2-2.5 h or 7-7.5 h post-cleavage. Before micromanipulation, embryos were exposed to a 0.1% pronase solution for 3 minutes to remove the zona pellucida, followed by disaggregation of blastomeres using a fine bore pipette.

Nuclear transfer

All micromanipulations were performed in Hepes-buffered CZB medium containing 1 μg ml^{-1} cytochalasin D (Sigma) and 0.1 μg ml^{-1} of nocodazole. Before enucleation, metaphase host cytoplasms were incubated for 5 min with the DNA vital stain, bisbenzimide (Hoechst 33342, 2 μg ml^{-1}, Sigma) and a small volume of the cytoplasm surrounding the metaphase plate was removed. Cytoplasmic fragments were exposed to ultraviolet irradiation to verify enucleation. A blastomere was immediately injected into the perivitelline space of the enucleated recipient and the membranes were fused by electrostimulation consisting of a 1.5 kV electric pulse for 60 μsec in a 0.3 M mannitol solution containing 0.1 mM $MgSO_2$ and 0.05 mM $CaCl_4$. After electrofusion, reconstructed oocytes were washed and cultured in bicarbonate-buffered CZB medium until fixation. Telophase host cytoplasts were selected by the presence of the second polar body at 1.5 h post-activation and stained with Hoechst dye. The second polar body and a small portion of the surrounding cytoplasm were removed and exposed to ultraviolet irradiation to confirm enucleation. Electrofusion with donor blastomeres was performed 3 h post activation. Interphase host cytoplasms were selected by the presence of a pronucleus at 6.5 h post-activation, enucleated without chromatin staining, and used for fusion at 8 h post-activation. When donor nuclei were obtained at metaphase before cleavage to 8-cells, only a karyoplast consisting of the chromatin and part of the blastomere cytoplasm were transferred to the host cytoplasm. When the donors were 8-cell interphase nuclei at G1/early S and late S/early G2, however, the entire blastomere was transferred.

Bovine oocyte source and manipulation

Bovine oocytes were obtained by follicular aspiration from slaughterhouse-derived ovaries. Follicles with diameters between 2 and 8 mm were punctured with a 19-gauge needle and cumulus-oocyte-

complexes (COCs) with several layers of cumulus cells and homogeneous oocyte cytoplasm were washed in Hepes-buffered tissue culture medium (TCM-199; Gibco BRL, Burlington, Canada) supplemented with 10% (v/v) fetal calf serum (FCS, Gibco). Groups of 20 COCs were placed in 100 µl of bicarbonate-buffered TCM-199 supplemented with 10% FCS, 50 µg ml^{-1} LH (Ayerst, London, ON, Canada), 0.5 µg ml^{-1} FSH (Folltropin-V; Vetrepharm, St-Laurent, PQ, Canada), 1 µg ml^{-1} estradiol 17-ß (Sigma, St-Louis, MO, USA), 22 µg ml^{-1} pyruvate (Sigma) and 50 µg ml^{-1} gentamicin (Sigma). Oocytes were denuded of cumulus cells after 24 h of maturation, selected for the presence of the first polar body, and micromanipulated in PBS containing 7.5 µg ml^{-1} cytochalasin B (Sigma). Metaphase II oocytes were enucleated by removing approximately 30% of the cytoplasm adjacent to the first polar body, placed in medium containing 5 µg ml^{-1} Hoechst 33342 for 10 min and exposed briefly to ultraviolet irradiation to verify by the absence of chromatin that enucleation was complete. Telophase and interphase oocytes were exposed to an activation stimulus with ionomycin (Sigma) and returned to culture to allow for extrusion of the second polar body. After 2 hours, activated oocytes were enucleated by removing a small portion of the cytoplasm adjacent to the second polar body (Bordignon, Smith, 1998). Oocytes to be reconstructed at interphase returned to the culture for an additional 5 h before nuclear transfer and electrofusion.

Immunocytochemistry

Groups of nuclear-transfer reconstructed oocytes were fixed in 10% formalin (Sigma) for 20 min, washed and stored at 4°C in 0.9% saline containing 0.1% Tween-20. To detect somatic histone H1, fixed oocytes were incubated in a blocking solution (PBS, 3% BSA, 0.5% Triton X-100) for 1 hr at room temperature, then transferred to anti-histone H1 antibody (raised in rabbit using histone H1 from rat thymus and affinity-purified (Bustin, Stollar, 1973; Sluyser, Bustin, 1974; Clarke et al. 1992)

diluted in 1:50 in blocking solution, and incubated overnight at 4°C. This antibody has previously been shown to recognize somatic H1 subtypes but not H1 subtypes present in mouse and bovine oocytes and early embryos (Clarke et al. 1992; Smith et al. 1995). The cells were then washed twice in blocking solution, incubated in fluorescein-conjugated goat (anti-rabbit) IgG diluted 1:100 in blocking solution for 1 hr at room temperature, and washed as above. Specimens were mounted on slides in a mounting medium containing Mowiol (Hoechst), the DNA stain DAPI (1 μg ml^{-1}, Sigma) and the anti-fading agent, DABCO (Sigma). Oocytes and embryos were examined using standard epi-fluorescence optics. Nuclear diameters were measured using an ocular micrometer under 400 X magnification.

Drugs used to inhibit cellular activities

Reconstructed oocytes were exposed to inhibitors of DNA replication (aphidicolin, 100 μg ml^{-1}; Boehringer), transcription (α-amanitin, 1.5 μM; Boehringer), protein synthesis (cycloheximide, 10 μg ml^{-1}; Sigma), protein phosphorylation (6-DMAP, 3 mM; Sigma), mitchondrial electron transport or glycolysis (*p*-trifluoromethyloxyphenylhydrazone [FCCP], 1 μM, Sigma and 2-deoxyglucose [2-DG], 6 mM, Sigma), histone deacetylase (trichostatin A, 100 nM; Biomol) and proteolysis (E-64, 10 μg ml^{-1}; Boehringer), before being fixed and processed for immunofluorescence.

Statistical analysis

Analysis of nuclear diameters were performed by analysis of variance using the Tukey-Kramer HSD test, with the significance level set at 5%. Frequencies of somatic H1 staining among groups of nuclear-transfer embryos were analyzed by chi-square.

Results

Host and donor cell cycle stage affect H1 remodeling

The main objective of the first experiment was to assess the effect of cell cycle stages to modify the somatic histone H1 content of chromatin using a combination of three different stages of both donor nuclei and recipient cytoplasts (Figure 1). Initially, G1/early S nuclei were fused to host cytoplasm from enucleated secondary oocytes at either metaphase, telophase (3 h post activation) or at interphase (8 h post activation). Reconstructed embryos were fixed at 1, 2, 4, 8, 16 and 20 hours post fusion and immunocytochemicaly processed to detect the presence of the somatic H1. Donor nuclei at metaphase and at late S/G2 were also transferred to the above different cytoplasm stages, however, based on the results observed in the previous group, fixation and immunocytochemistry was just performed at 2, 4 and 16 h post fusion. Our main finding in this experiment was that most reconstructed embryos lose the somatic H1 staining within a short period after fusion and the loss of somatic H1 immunoreactivity is affected by the cell cycle stage of both donor nuclei and host cytoplasts (Figure 2; Table 1). Independently of the cell cycle stage of donor nuclei, fusion to enucleated host cytoplasts at metaphase stage led to a complete loss of the somatic H1 immunoreactivity within few hours after reconstruction (Figure 3). Although telophase stage enucleated cytoplasts also induced a loss of the somatic H1 immunoreactivity of nuclei transferred at any stage of the cell cycle, the time elapsed to complete loss of the staining was slightly longer than that observed with metaphase cytoplasts. Moreover, telophase cytoplast groups were particularly prone to induce an accelerated reappearance of immunoreactive to somatic H1. Whereas the number of H1 stained nuclei was practically absent at 4 h after fusion, a very large proportion of nuclei were immunoreactive to somatic H1 by 16 h after fusion, earlier than expected for control fertilized zygotes that become

immunoreactive after cleavage to the 2-cell stage. Contrary to the results observed with metaphase and telophase cytoplasts, interphase cytoplasts were mostly unable to induce a loss of somatic H1 immunostaining in transplanted nuclei. With the exception of late S/G2-stage nuclei that remained positive throughout the fixation period, metaphase and G1/earlyS-phase nuclei were largely unstained at 16 h after fusion. These results confirm our previous findings with bovine reconstructed embryos, where the timing of loss of somatic H1 immunoreactivity was increasingly longer in nuclei fused later after activation (Bordignon et al. 1999).

The effect of donor blastomere cell cycle stage on the timing when somatic H1 became undetectable on the chromatin of transferred nuclei was apparent but less dramatic than that observed among cytoplast groups (Figure 3). Since metaphase cytoplasts invariably led to a rapid loss of H1 staining, most of the donor nuclear effects on the timing of somatic H1 immunoreactive loss were observed among the telophase and interphase cytoplast groups. Metaphase nuclei lost their somatic H1 staining faster and more completely than G1/early S- and late S/G2 nuclei. None-the-less, metaphase nuclei remained somatic H1-positive at 2 h after transfer, indicating that, even in the absence of a nuclear envelope, interphase cytoplasts were unable to modify the chromatin's immunoreactivity to somatic H1 before 4 h after fusion. Moreover, none of the nuclei at late S/G2 stage lost their somatic H1 immunoreactivity after transplantation into interphase stage cytoplasts, indicating an inability of interphase cytoplasm to remodel the H1 component of chromatin that have already undergone DNA replication. Together, these findings show that the timing and degree of the loss and assembly of immunoreactive somatic histone H1 after nuclear transplantation are affected by the cell cycle stage of both donor nuclei and host cytoplasts.

To verify whether the loss of somatic H1 immunostaining was correlated with the swelling rate of transplanted nuclei, the diameter of G1/early S stage donor nuclei was measured at different time after fusion

to metaphase, telophase and interphase cytoplasts (Figure 4). At 1 h after fusion, all group diameters differed significantly from each other, indicating an immediate effect of the host cytoplast on nuclear morphology. Although nuclei fused to metaphase and telophase cytoplasts swell continuously during the first four hours after fusion nuclei fused to intephase cytoplasts retained the same diameter and were significantly smaller by 8 h after fusion. Although there seems to be a relationship between swelling and the disappearance of somatic H1 immunostaining, metaphase cytoplasts induce a faster loss of H1 immunostaining and a more more gradual swelling when compared to telophase cytoplasts. No relationship is apparent in the interphase cytoplast group. Together, these results suggest a poor or absent biological relationship between the swelling phenomena and the H1 chromatin remodeling. Technically, these results indicate that the degree of staining to our somatic H1 antibody is not dependent on the volume of the nucleus, excluding the possibility that the loss of immunoreactive histone H1 might be linked to nuclear swelling.

Pronuclear enucleation influences remodeling

This experiment was performed with the objective of verifying whether the assembly of a pronucleus and its eventual removal during enucleation were involved in the inability of interphase stage host cytoplasts to modify the immunoreactivity of transplanted nuclei to somatic H1. Blastomeres at the G1/early S stage were fused to 8 h post activation interphase host of one of the following three origins. First, host cytoplasts were enucleated at 1.5 h post activation, returned to culture until blastomere fusion at 8-h post activation. Second, pronuclear stage eggs were enucleated at 7-h post activation followed by blastomere fusion within the following hour. Finally, a third host cytoplasm group had only the polar body removed from pronuclear stage eggs and fused to a blastomere at 8-h post activation (non-enucleated controls).

Reconstructed oocytes were fixed at 2 and 4 h post fusion to detect somatic H1. Embryos reconstructed with non-enucleated controls showed a negative immunoreactive endogenous pronucleus and mostly positively stained donor nuclei at both 2 (89%) and 4 h (96%) post reconstruction (Figure 5). As shown in the previous experiment (Figure 2), cytoplasts enucleated at pronuclear stage (8 h post activation) were also mostly positively stained at 2 h (94%) and had a slightly fewer positive stained donor nuclei at 4 h (68%) post fusion. However, nuclei transferred to interphase cytoplasts that had been enucleated at telophase stage (1.5-3 h post activation) and returned to culture until 8 h post-activation, showed significantly fewer reconstructed embryos with donor nuclei showing positive somatic H1 immunostaining at 2 h (43%) and 4 h (28%) after fusion. These results indicate that at least part of the inability of interphase stage cytoplasts to cause donor nuclei to lose their somatic H1 immunostaining after reconstruction is due to pronuclear enucleation, suggesting that the assembly of a pronucleus and its subsequent removal depletes the host cytoplasm of factors required to modify the immunoreactivity of donor nuclei to somatic H1. The assembly of a pronucleus in the host cytoplasts was also correlated with the swelling rate of the donor nucleus since nuclei transplanted into cytoplasts enucleated at telophase II stage showed a rapid and pronounced swelling whereas those transplanted into eggs enucleated at the pronuclear stage and non-enucleated pronuclear controls had significantly smaller diameters at 4 h after fusion ($P<0.05$).

Interspecies somatic H1 remodeling

Previous studies with bovine reconstructed embryos had shown that nuclei from morula-stage blastomeres were modified in a fashion dependent on the cell cycle stage of host cytoplasts (Bordignon et al. 1999). Our objective in this experiment was to investigated whether the mechanisms operating within species to modify the immunoreactivity of

donor chromatin to somatic H1 were functional across species barriers. Mouse blastomeres at G1/early S stage were fused to enucleated bovine secondary oocytes at the metaphase, telophase (3-h post activation) or interphase (8-h post activation) stages (Table 2). All the bovine cytoplasts tested were able to modify the somatic H1 immunostaining of mouse transplanted nuclei within few hours after electrofusion. Moreover, a clear effect of the cell cycle stage of the cytoplasts was observed since H1 staining was lost more rapidly from mouse donor nuclei fused to bovine cytoplasts before or soon after oocyte activation. While nuclei transferred to metaphase cytoplasts were completely unstained within 1 h post fusion, those transferred to telophase and interphase stage cytoplasts completed the staining loss by 2 and 4 h post fusion, respectively. Furthermore, it appears that bovine cytoplasts are able to modify the immunoreactivity of murine nuclei to somatic H1 even faster since interspecific reconstructed embryos showed no H1 staining of donor nuclei at least 2 h earlier after fusion to each cytoplast groups (Table 1). These results indicate that the mechanism controlling the loss of somatic H1 in transplanted nuclei are not species-specific among mammals and that the effects caused by the cell cycle stage operate at least as effectively across species.

Effect of inhibitory agents on chromatin remodeling

Reconstructed embryos were exposed to several metabolic inhibitory agents to investigate the molecular mechanisms implicated in the loss of somatic H1 immunoreactivity after nuclear transfer. Blastomeres at the G1/early S stage were fused to metaphase stage cytoplasts, cultured for 4 h in the presence of the different inhibitors then fixed and immucytochemically processed for somatic H1. Control embryos cultured in the absence of inhibitory agents using this protocol underwent complete loss the somatic H1 immunostaining when analyzed at 4 h post electrofusion. Apart from some exceptions, most reconstructed

embryos exposed to 6-DMAP (0/25), FCCP+ 2-DG (1/12), trichostatin A (1/14) and E-64 were negatively stained to somatic H1, indicating that kinase phosphorylation, energy-dependent nuclear transport and acetylase activity were not required for the loss of somatic H1 staining, respectively. However, a small proportion of reconstructed embryos exposed to cycloheximide (4/23) and aphidicolin (3/14) remained positively stained at 4 h, indicating that both protein synthesis (cycloheximide) and DNA replication (aphidilcolin) may be partially involved in the loss of somatic H1 immunoreactivity. These results contrast with our previous findings in the bovine model where both 6-DMAP and cycloheximide had a more potent effect on blocking the loss of H1 staining in that species (Bordignon et al. 1999).

Discussion

Developmentally regulated structural modifications are probably involved in the control of chromatin activity during embryogenesis and cellular differentiation, and might determine important drawbacks in the functional reprogramming of nuclei transplanted into enucleated oocytes. In this study we have used an antibody that recognizes specifically somatic forms of the linker histone H1 to investigate whether somatic H1-positive transplanted nuclei are remodeled into pronucleus-like somatic H1-negative nuclei by contact with host cytoplasts and to what extent the cell cycle stages of donor nuclei and host cytoplasts influence the kinetics and degree of chromatin remodeling after nuclear transfer. We demonstrate that most immunoreactive blastomere nuclei lose the somatic H1 staining before completing the first cell cycle resembling a pronucleus in both size and absence of somatic H1 staining. These results resemble findings in bovine reconstructed embryos that lost somatic H1 immunoreactivity within a short period after embryo

reconstruction, indicating similarities in chromatin remodeling among widely diverse mammalian species (Bordignon et al. 1999). It is tempting to speculate that the somatic H1 component of donor chromatin needs to be remodeled in order to reset the developmental program back to the totipotent-state of single cell zygotes.

Implication of the host and donor cell cycle stage on the immunoreactivity of donor chromatin to somatic histone H1

Three different stages of donor nuclei and host cytoplasts were combined to determine the effects of cell cycle on the remodeling of structural components of the chromatin after nuclear transplantation. By monitoring changes in the immunoreactivity of donor chromatin to somatic H1, we demonstrate that different donor:host combinations led to contrasting patterns in the remodeling of somatic H1. Whereas metaphase nuclei fused to metaphase cytoplasts led to a complete loss of immunoreactive somatic H1 from donor chromatin within less than two hours after fusion, S/G2-phase nuclei fused to interphase cytoplasts were unable to remodel the chromatin in any reconstructed embryo throughout the first or latter cell cycles. These results support previous findings indicating that metaphase cytoplasts have improved ability to remodel donor chromatin. In amphibians, oocytes and eggs differ in several aspects in remodeling chromatin and the development potential of reconstructed embryos is improved by the use of metaphase stage cytoplasts (DiBerardino, Hoffner Orr, 1992). In mammals, thesecondary ooplast is important for the remodeling of sperm, blastomere and somatic donor chromatin (Czolowska et al. 1984; Szöllösi et al. 1988; Borsuk, Tarkowski, 1989; Collas, Robl, 1991; Barnes et al. 1993; Borsuk et al. 1996; Smith et al. 1996). Overall, these studies indicate that cytoplasts from secondary oocytes can induce a better remodeling of transplanted chromatin, including a pronuclear-like size by promoting faster nuclear swelling and the resetting of the pronuclear-like chromatin functions. Our

data on somatic H1 immunoreactivity extend these observations by showing that not only host cytoplast but also donor nuclei in metaphase are more effective in remodeling the structural components of transplanted chromatin. Although donor nuclei at metaphase were remodeled more rapidly than nuclei in G1/early S and late S/G2 stage, only a proportion of nuclei lost the somatic H1 immunoreactivity after transplantation into interphase cytoplasts, suggesting that the remodeling of transferred chromatin resides more on the host cytoplasts than the nuclear cell cycle stage.

Despite a clear implication of the cell cycle stage on the remodeling of the somatic H1 on donor chromatin the mechanism involved in this remodeling remain unclear. It is possible that kinases implicated in the control of the cell cycle are involved. The high activity of the M-phase promoting factor (MPF) at the metaphase stage will induce the breakdown of the nuclear membrane and chromatin condensation (Nurse, 1990). It has been shown that nuclear envelope permeabilization allows accessibility to cytoplasmic factors capable of modifying chromatin activity (Blow, Laskey, 1988; Leno et al. 1992). Moreover, nuclear membrane breakdown is correlated with the structural and functional remodeling of somatic nuclei transplanted into mouse oocytes (Szöllösi et al. 1998). Thus, it might be speculated that nuclear envelope permeabilization by kinases present in metaphase cytoplasts could allow access to factors involved in the loss of somatic H1 staining. However, other evidences suggest that the absence of a nuclear membrane may not be the only component involved. First, several metaphase stage nuclei transferred to interphase cytoplasts remained positive to somatic H1 staining regardless of the lack of a nuclear membrane. Second, telophase stage host oocytes (3-h post activation) remodelled the somatic H1 component of transplanted nuclei, independent of their cell cycle stage. As previously shown in different species, MPF activity decays promptly after oocyte activation (Collas et al. 1993; Szöllösi et al. 1993; Collas et

al. 1995; Bordignon, Smith, 1998) and, therefore, is unable to affect the integrity of the nuclear membrane when nuclei are transplanted 1 h after oocyte activation (Borsuk et al. 1996; Szöllösi et al. 1998). Together this information suggest that cell cycle effect on the remodeling of the somatic H1 is probably not directly controlled by the effect of MPF on nuclear membrane or chromatin.

Another possibility is the direct effect of the cell cycle kinases on somatic H1. For example, it has been shown that the phosphorylation patterns of histone H1 can affect its stability within the chromatin (Hill et al. 1991; Aubert et al. 1991), indicating that kinases present in host cytoplasts sooner after activation could affect H1 displacement by changing its phosphorylation state. In amphibia, the replacement of nuclear proteins required for the remodeling of the sperm chromatin (Philpott, Leno, 1992) and the removal of the somatic H1 from nuclei relies on the action of the chaperone protein nucleoplasmin (Dimitrov, Wolffe, 1996). Moreover, the action of nucleoplasmin seems to be dependent of its phosphorylation state since hyperphosphorylated nucleoplasmins decondenses sperm chromatin and remove basic protein faster than in a hypophosphorylted state (Leno et al. 1996). Although a similar mechanism has not yet been shown in mammals, the involvement of nucleoplasmin in chromatin remodeling is a plausible hypothesis. It is possible that the cytoplasmic mechanism implicated in the remodeling of the chromatin is activated by metaphase stage kinases and that, once activated, this mechanism does not require de-novo phosphorylation to remain fuctional. This may explain the inability of 6-DMAP treatment to inhibit the capacity of metaphase cytoplasts to remodel somatic H1 in transplanted nuclei.

The involvement of the host cell cycle stage was also seen after transplantation of mouse nuclei into bovine cytoplasts. Although a complete loss of somatic H1 immunoreactivity occurred to all cytoplast groups tested, metaphase stage cytoplasts were more rapidly modified.

Beyond confirming the importance of the cell cycle stage, these results provide a molecular evidence that chromatin from one species can be remodeled after transplantation into a host cytoplast from widely appart species, supporting previous findings showing the ability of somatic nuclei from several species to support development to the blastocyst stage after transplantation to bovine enucleated oocytes (Dominko et al. 1998).

Effect of pronuclear assembly and removal on the remodeling of transplanted nuclei

We investigated whether the inability of interphase host cytoplasts to modify the immunoreactivity of somatic H1 after nuclear transplantation could be affected by the assembly followed by the removal of female pronucleus from host oocytes. Interphase cytoplasts enucleated before pronuclear formation induced the loss of the immunoreactive somatic H1 in a high proportion of transplanted nuclei. This contrasted with a clear inability of interphase cytoplast to remodel somatic H1 when pronuclei were assembled and either removed or left within the host cytoplats. It is possible that cytoplasmic factors required for the remodeling of transplanted chromatin are sequestered into the pronucleus leading to their depletion in pronuclear-enucleated and non-enucleated zygotes. In fact, as shows in the figure 4, nuclei transferred into interphase cytoplasts do not swell, contrasting to the rapid volume increase observed in nuclei fused to metaphase and telophase enucleated cytoplasts. It is tempting to speculate that the putative embryonic subtype of the histone H1 is assembled into the endogenous pronucleus and, thereby, depleting the cytoplasm of a replacement protein to replace the somatic H1 from donor chromatin. However, somatic H1 types are disassembled from chromatin exposed to egg extracts depleted of B4, suggesting that an embryonic form of H1 might not be absolutely necessary for replacing the somatic H1 from donor chromatin (Dimitrov, Wolffe, 1996). Whether or not the modifications in the immunostaining to

somatic H1 requires a replacement protein, swelling and activity of the transplanted nuclei are dependent on protein exchanges between the donor nuclei and host cytoplasts (Merriam, 1969; Blank et al. 1992), suggesting that the loss of immunoreactivity to somatic H1 indicates the involvement of a replacement protein in the exchange.

Effect of cellular metabolic inhibitory agents on the remodeling of transferred nuclei

Several cellular metabolic inhibitory agents were used to investigate the mechanisms implicated in the loss of immunoreactivity for somatic H1. Reconstructed embryos were cultured in the presence of different drugs during 4 h, a period sufficient to induce a complete loss of somatic H1 immunostaining in the control group. Although none of the treatments were able to completely prevent the loss of somatic H1 staining, inhibition of DNA replication with aphidilcolin inhibited the loss of staining in 21 % (3/14) of transferred nuclei. Although DNA replication is required for the assembly of somatic H1 during early embryogenisis in mice and bovine (Clarke et al. 1992; Smith et al. 1995), aphidilcolin did not affect the loss of somatic H1 immunostaining after bovine nuclear transplantation when using non-synchronized morula-stage donor nuclei fused to telophase enucleated oocytes (Bordignon et al. 1999). These contrasting results point towards the important role the cell cycle stage of the donor nucleus seems to play on the effect of inhibitory agents after nuclear transfer. However, G1-phase nuclei may be particularly permissive to chromatin remodeling regardless of inhibitory agents, as indicated by the inability to block the loss of somatic H1 immunostaining after transfer of G1-synchronized morula-stage nuclei in the presence of cycloheximide and 6-DMAP (Bordignon et al. 1999). None-the-less, 17% (4/23) of mouse G1-phase nuclei remained positively stained at 4 h post transplantation in the presence of cycloheximide, suggesting a role for of protein synthesis in the process of H1 remodeling. A similar inhibitory

effect cycloheximide was observed with bovine embryos reconstructed with non-synchronized blastomeres but with G1-phase nuclei (Bordignon et al. 1999). At this point it is unclear whether protein synthesis is required for either the displacement of somatic H1 form, for the replacement of a putative embryonic form of H1 or both.

Most of reconstructed embryos cultured in the presence of FCCP, an inhibitor of mitochondrial electron transport, and 2-DG, an inhibitor of glycolysis, lost the somatic H1 staining within 4 h, indicating that a cellular energy supply is not required to induce the immunoreactive modifications to somatic H1 on donor chromatin. This finding contrasts with previous reports showing that the transport of the somatic H1 into the nucleus is dependent of energy supply (Breeuwer, Goldfarb, 1990; Kurz et al. 1997), suggesting that somatic H1 transport and displacement are not under the same control. Although the pattern of core histone acetylation plays an important role in the control of chromatin transcription (Grunstein, 1997) and the inhibition of histone deacetylation interferes with the expression of different genes, including a higher expression of the linker histone $H1^0$ gene (Almouzni et al. 1994; Girardot et al. 1994), the exposure of reconstructed embryos to trychostatin A, an inhibitor of histone deacetylase, was not effective in preventing the loss of somatic H1 immunostaining. Culture of reconstructed embryos in the presence of E-64, an inhibitor of proteolysis activity, did not prevent the remodeling of the somatic H1 from transplanted nuclei, indicating that the loss immunostaining is not dependent of protein degradation.

Although the molecular mechanism underlying the remodeling of the somatic histone H1 in donor nuclei after nuclear transplantation remains unclear, the results obtained in the present study show a clear implication of the cell cycle stage. Considering the possible role of somatic H1 in chromatin activity, it might be speculated that cell cycle stage of donor nuclei and host cytoplasts at time of reconstruction regulates

important structural and functional aspects of the chromatin that may be required for the resetting of the developmental program following the transfer of nuclei into oocyte cytoplasts.

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Table 1. Effect of donor and host cell cycle stage on the immunostaining for somatic histone H1 in chromatin at different times after nuclear transplantation

		Cell cycle stage of recipient ooplast at time of fusion								
		metaphase II			telophase II (3 h)			Interphase (8 h)		
Donor nuclei stage	Time fixed (h post fusion)	n	+	-	n	+	-	n	+	-
G1/S	1	8	3	5	5	4	1	6	6	0
	2	9	1	8	10	6	4	7	7	0
	4	9	0	9	7	0	7	8	7	1
	8	5	0	5	5	0	5	8	8	0
	16	7	0	7	7	5	2	10	5	5
	20	4	4	0	7	7	0	6	4	2
M	2	9	0	9	11	1	10	16	12	4
	4	9	0	9	12	0	12	13	4	9
	16	9	5	4	12	12	0	13	5	8
S/G2	2	10	1	9	9	9	0	8	8	0
	4	9	0	9	9	1	8	10	10	0
	16	10	0	10	10	10	0	11	11	0

Table 2. Loss of immunoreactive somatic H1 of G1/early S 8-cells stage mouse blastomeres transferred into bovine ooplasts at different stages of the cell cycle.

	Cell cycle stage of recipient ooplast at time of fusion								
time fixed	Metaphase II			Telophase II (3 h)			Interphase (8 h)		
(h post-fusion)	n	+	-	n	+	-	N	+	-
1	7	0	7	9	7	2	6	3	3
2	8	1	7	9	0	9	7	5	2
4	5	0	5	6	0	6	4	0	4
12	5	0	5	6	0	6	9	0	9

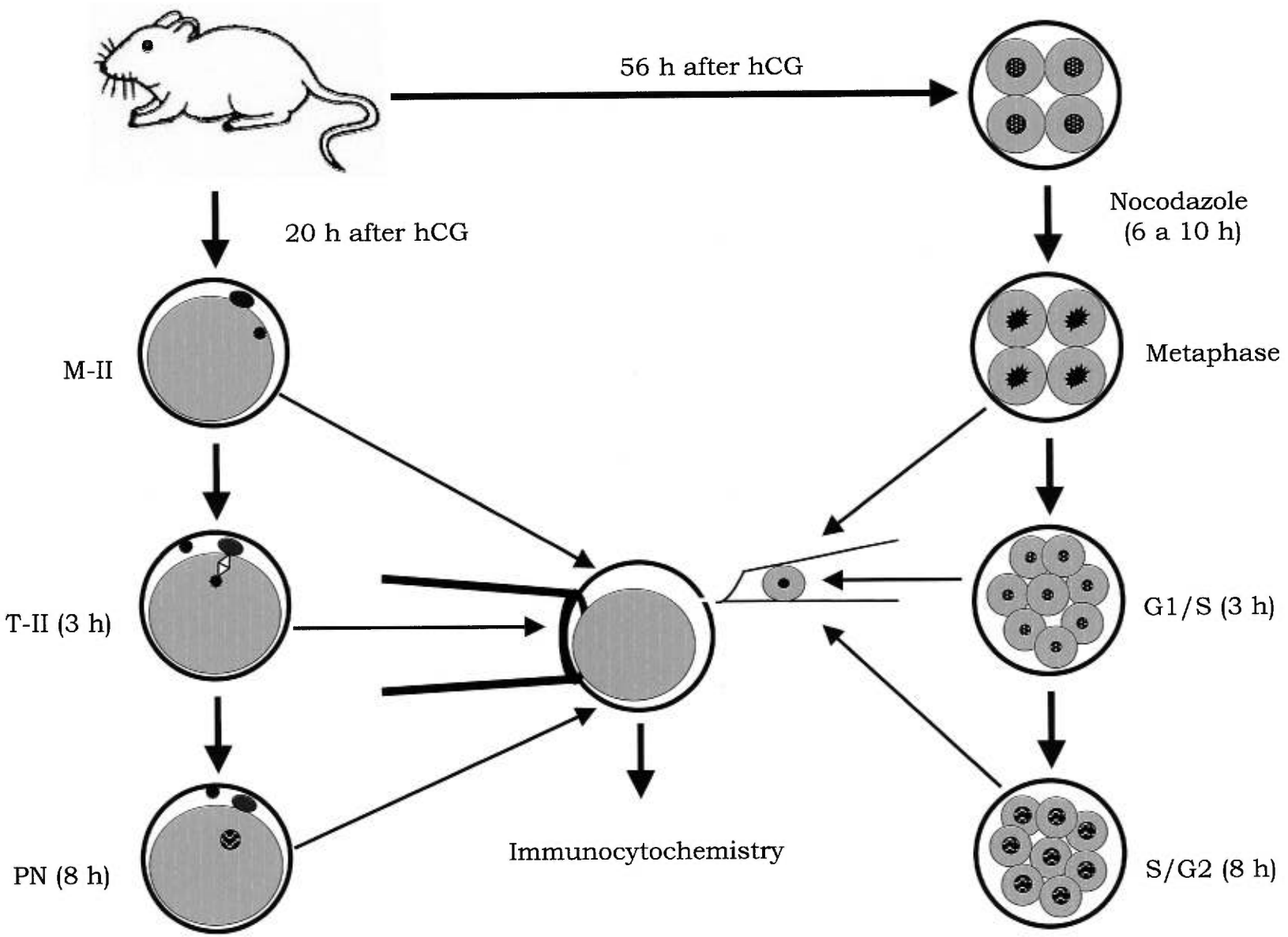
56 h after hCG
20 h after hCG
Nocodazole
(6 a 10 h)
M-II
Metaphase
T-II (3 h)
G1/S (3 h)
PN (8 h)
Immunocytochemistry
S/G2 (8 h)

Figure 1.

Schematic representation of the experimental protocol used to reconstruct mouse embryos. Host oocytes from superovulated females were used between 20 to 24 post hCG injection either at metaphase or activated and used at telophase II (3 h post activation) or interphase stage (8 h post activation). Donor nuclei embryos were recovered at 4-cells stage at 56 h post hCG, cultured in the presence of nocodazole (6 – 10 h) and used at metaphase or removed of nocodazole and used after 3 or 8 h. Reconstructed embryos were fixed and immunocytochemicaly processed to detected the presence of the somatic H1 at several different times.

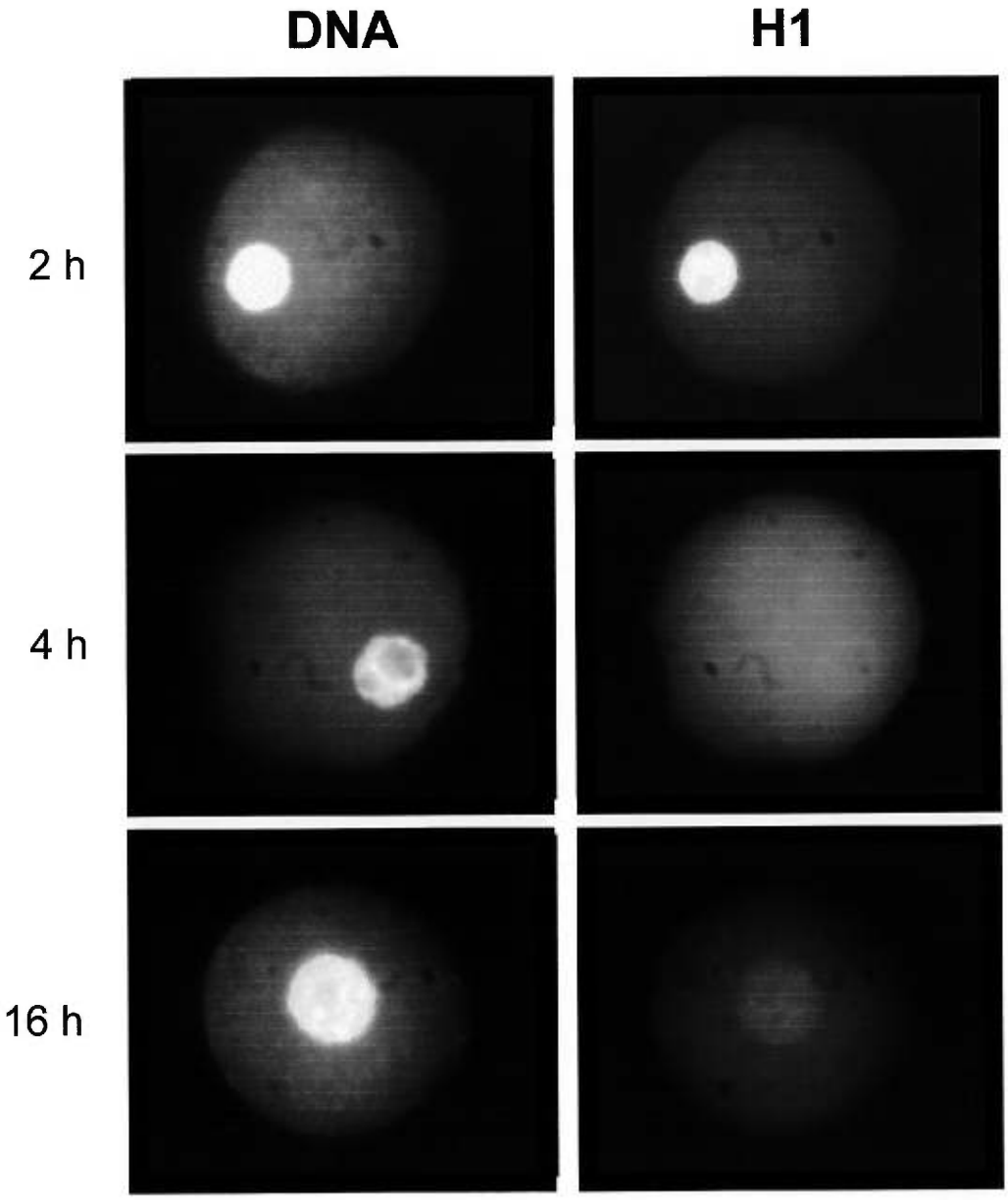
DNA
H1
2 h
4 h
16 h

Figure 2.

Immunostaining (FITC) showing the remodeling of the somatic histone H1 into the chromatin of reconstructed embryos (right panel) and DNA staining (DAPI, left panel). Representative examples of nuclei fused at G1/early stage (3 h post nocodazole) with host cytoplasts at T-II (3 h post activation) fixed at 2, 4 and 16-h post reconstruction. 400 X magnification.

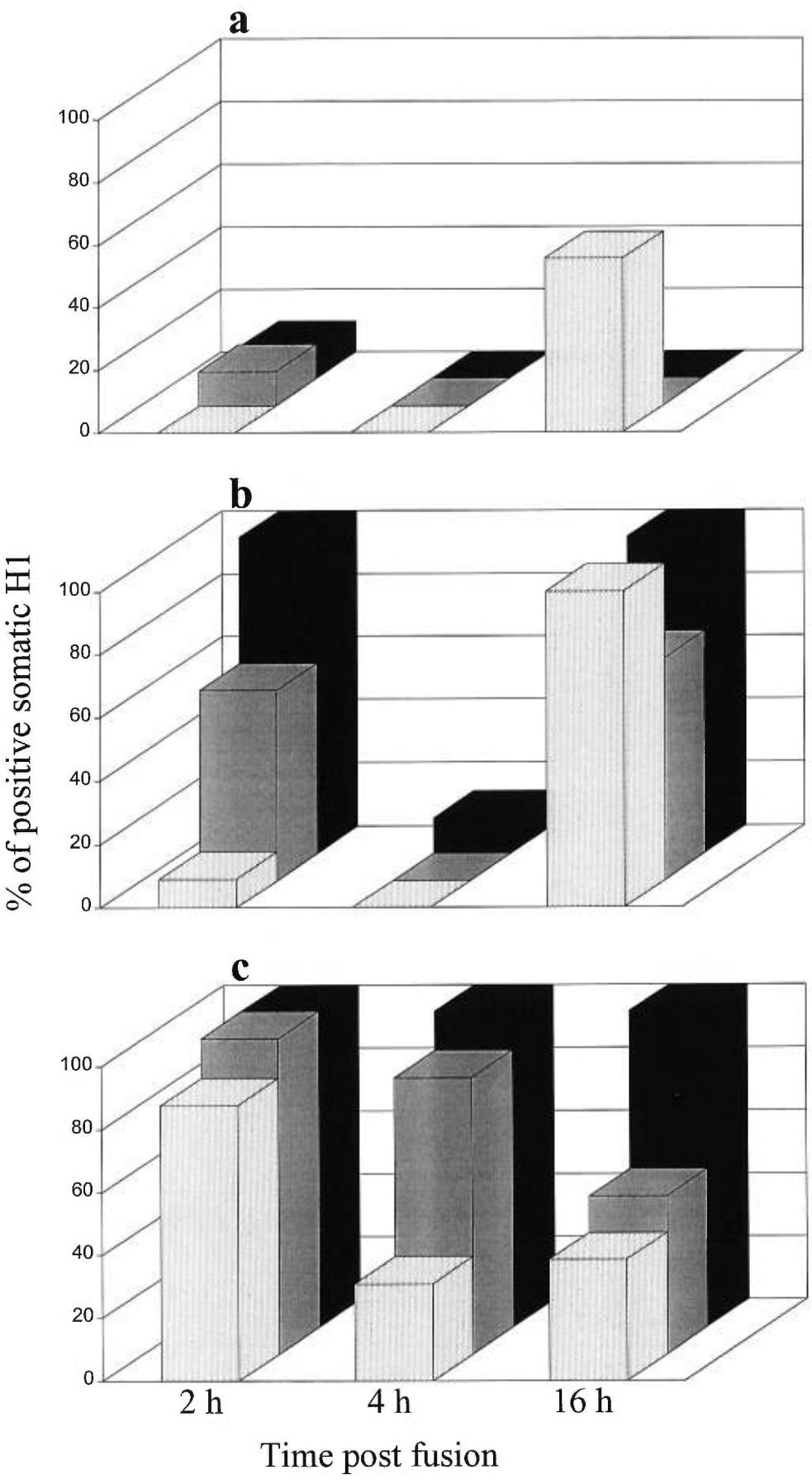
a
b
c
100
80
60
40
20
0
% of positive somatic H1
2 h
4 h
16 h
Time post fusion

Figure 3.

Effect of cytoplasts cell cycle stage on the remodeling of somatic histone H1 after nuclear transplantation. Bars represent frequencies of somatic histone H1 negative stained nuclei transplanted to metaphase (a), telophase (b) or interphase (c) cytoplasts. Nuclei were transferred at metaphase (light gray), G1/eraly S (dark gray) or late S/G2 (black) stage.

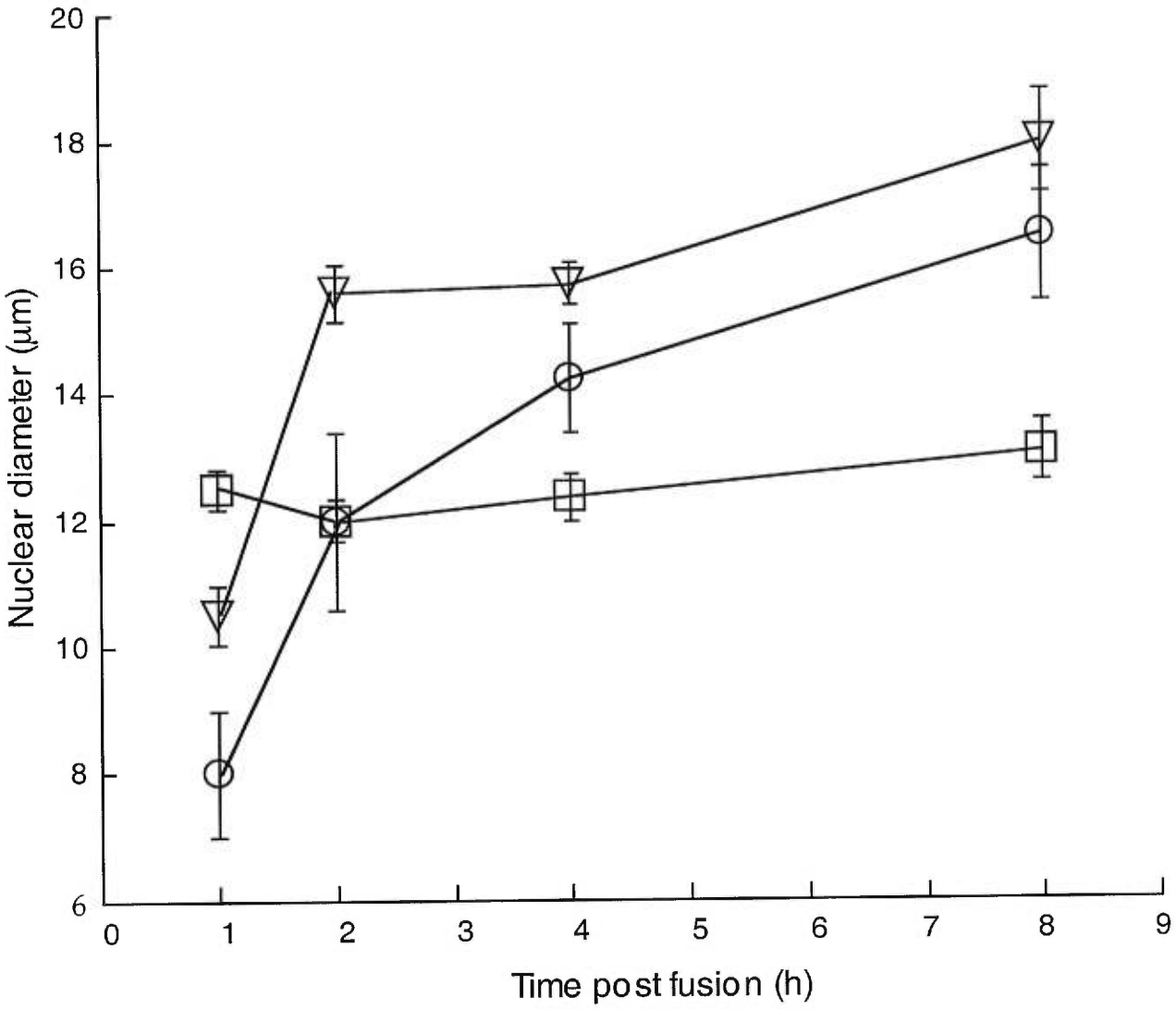
20
18
16
14
12
10
8
6
Nuclear diameter (µm)
0
1
2
3
4
5
6
7
8
9
Time post fusion (h)

Figure 4.

Changes in the diameter of G1/early S stage nuclei after transplantation into cytoplasts at different stages of cell cycle. Means and standard errors (vertical bars) of nuclear diameter between 1 and 8 h after fusion with metaphase (circle), telophase (triangle) and interphase (square) stage cytoplasts.

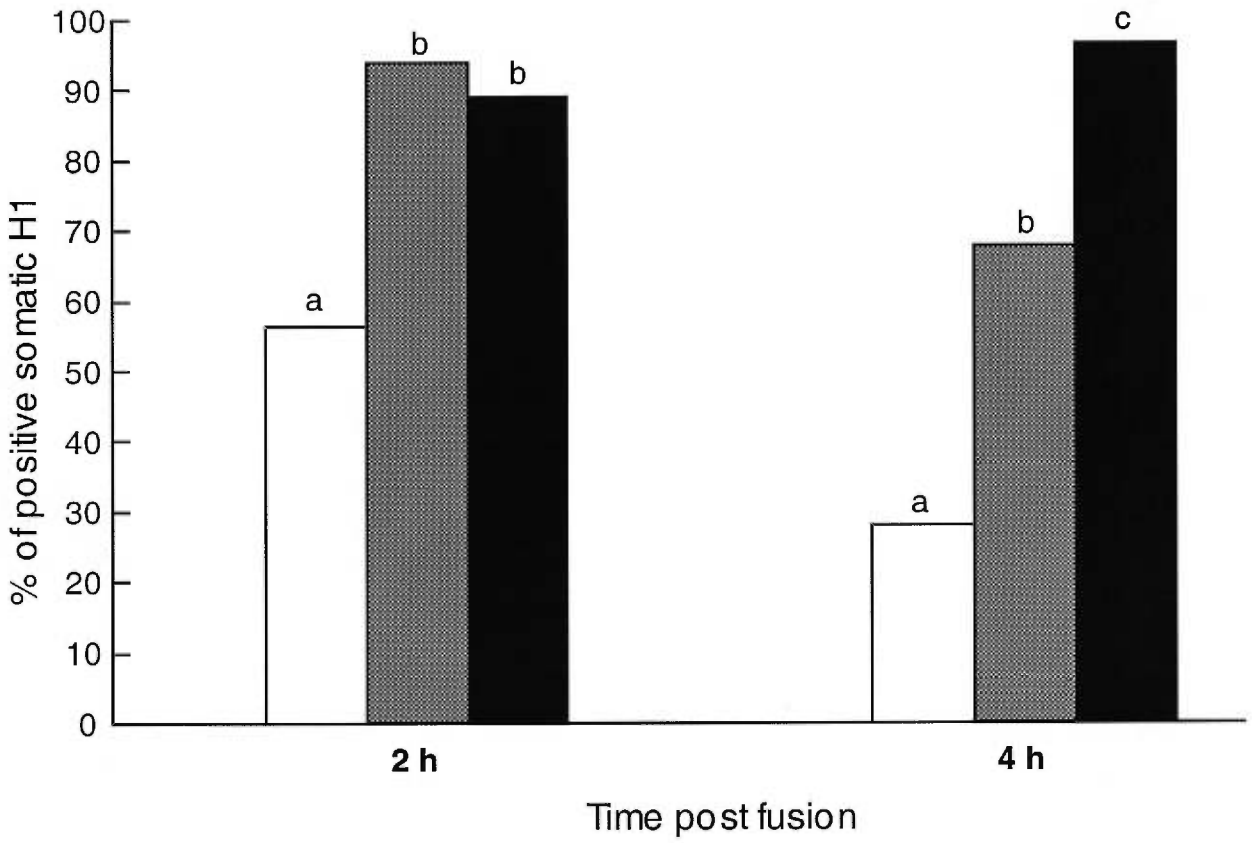
100
90
80
70
60
50
40
30
20
10
0
% of positive somatic H1
a
b
b
a
b
c
2 h
4 h
Time post fusion

Figure 5.

Effect of pronuclear growth into the host cytoplasts before nuclear transfer on the remodeling of the somatic histone H1. Bars represent frequencies of positive stained nuclei transplanted to interphase cytoplasts encucleate before pronuclear growth (white), after pronuclear growth (gray) or non-enucleated (black).

VI. DISCUSSION GÉNÉRALE

Notre objectif était de mettre au point des techniques de transfert nucléaire qui permettraient d'obtenir un meilleur taux de développement des embryons reconstitués et de comprendre davantage les mécanismes qui commandent la reprogrammation des noyaux transplantés. Nous avons donc testé des procédures d'activation des ovocytes hôtes puis étudié le rôle du stade du cycle cellulaire des ovocytes hôtes et des noyaux transplantés sur le remodelage des composants structurels de la chromatine à la suite de la transplantation de noyaux provenant des blastomères.

La première partie du travail consistait à trouver une méthode efficace d'induction de l'activation des ovocytes bovins, qui serait par la suite utilisée pour la préparation des ovocytes hôtes en vue du clonage d'embryons bovins. Initialement, nous avons comparé l'effet de l'éthanol et celui d'une réduction de la température sur le taux d'activation (formation des pronoyaux) puis sur la réduction de l'activité H1 kinase (MPF) des ovocytes bovins mûris *in vitro* pendant 26 ou 30 h. Les résultats obtenus montrent que les taux de formation de pronoyaux sont supérieurs et l'activité MPF plus efficacement réduite chez les ovocytes traités à 30 h que chez ceux qui le sont à 26 heures. Ces résultats, soit qu'une plus longue période de maturation a un effet bénéfique sur la réponse des ovocytes à l'activation, concordent avec ceux de plusieurs autres études menées chez différentes espèces (Kubiak, 1989; Nagai, 1987; Ware et al., 1989). Ce phénomène est encore mal compris, mais il semble qu'une réduction de la synthèse du produit du gène c-mos (Wu et al., 1997a), un composant du facteur cytostatique (CSF) responsable du blocage des ovocytes en métaphase II (Lorca et al., 1993), soit en cause.

Nous avons montré que l'activation des ovocytes avec 7% d'éthanol produit un meilleur taux d'activation et une plus forte réduction de l'activité kinase H1 qu'une réduction de la température ou que

l'association des deux traitements. Comme plusieurs autres agents utilisés pour l'activation des ovocytes, l'éthanol provoque une augmentation intracellulaire de calcium (Shiina et al., 1993), mais il n'induit pas les nombreuses oscillations de calcium observées normalement après la fécondation (Fissore et al., 1992; Kline et Kline, 1992). Par contre, la réduction de la température peut induire des oscillations de calcium chez les ovocytes de rat (Ben-Yosef et al., 1995). En outre, d'autres études ont démontré que le refroidissement des ovocytes hôtes avant le transfert nucléaire favorise le développement (Heyman et al., 1994; Stice et al., 1994; Ectors et al., 1995; Zakhartchenko et al., 1995). Par conséquent, l'association entre l'éthanol et le refroidissement aurait dû être plus efficace pour activer les ovocytes bovins, mais cela ne s'est pas avéré. À la lumière de ces résultats, nous avons décidé que les ovocytes seraient activés après 30 h de maturation par une exposition de 5 minutes à une solution de 7% d'éthanol.

Par la suite, notre objectif a été de concevoir une méthode d'énucléation des ovocytes pré-activés qui permettrait d'utiliser seulement ceux qui ont répondu au traitement d'activation et se trouvent en télophase II, c'est-à-dire après l'expulsion du deuxième globule polaire. Nous avons démontré que la micro-aspiration d'environ 10 % du volume total du cytoplasme autour du site d'extrusion du deuxième globule polaire se traduisait par un taux d'énucléation de 98 % bien supérieur aux 59 % obtenus avec la technique standard (aspiration d'environ 30 % du cytoplasme autour du premier globule polaire). Sur la base des taux élevés de réussite, on peut affirmer que la méthode d'énucléation au stade de télophase permet d'obtenir des résultats fiables sans le recours à des colorants fluorescents ou à l'exposition aux rayons ultraviolets habituellement nécessaires pour confirmer la réussite de l'énucléation.

À la dernière étape de ce projet, nous avons comparé le potentiel de développement jusqu'au stade de blastocyste d'embryons reconstitués à partir d'ovocytes hôtes énucléés au stade de télophase (méthode *télophase*) à celui d'embryons produits à l'aide de la procédure standard de reconstitution des embryons bovins dite méthode des ovocytes *vieillis* (énucléation, vieillissement, refroidissement) (Heyman et al., 1994; Stice et al., 1994; Ectors et al., 1995; Zakhartchenko et al., 1995). Le taux de développement jusqu'au stade blastocyste (38 % contre 16 %) et le nombre de cellules par blastocyste (126 contre 84) sont plus élevés chez les ovocytes hôtes du groupe *télophase* que chez les ovocytes du groupe *vieillis*. Plusieurs raisons peuvent expliquer la supériorité de cette technique : 1) elle permet de sélectionner les ovocytes qui ont répondu au traitement d'activation et dont le deuxième globule polaire a été expulsé. Ce type de sélection est évidemment impossible si l'énucléation a été effectuée avant l'activation, 2) elle permet d'obtenir un taux élevé d'énucléation sans utilisation de colorants fluorescents ou exposition aux ultraviolets susceptibles d'endommager les ovocytes et de réduire leur potentiel de développement (Smith, 1993) et 3) elle permet d'énucléer les ovocytes sans prélever une fraction trop importante du volume de cytoplasme. En effet, une trop forte réduction du volume du cytoplasme peut affecter le développement et le nombre de cellules par embryon (Evsikov et al., 1990; Westhusin et al., 1996). Outre ces avantages, la technique *télophase* est simple, c'est pourquoi son utilisation se répandra probablement dans le domaine du clonage des embryons bovins.

Bien que la méthode *télophase* ait permis d'augmenter de manière satisfaisante le taux de développement des embryons reconstitués, les méthodes d'activation testées ont produit des résultats satisfaisants seulement lorsque les ovocytes avaient subi une période de maturation de 30 h. D'autres études ont par ailleurs montré que le taux de développement des ovocytes bovins fécondés *in vitro* décroît quand la

période de maturation dépasse les 26 h (Chian et al., 1992). Toute méthode capable d'activer des ovocytes plus jeunes devrait donc améliorer le potentiel de développement après transfert nucléaire. C'est pourquoi nous voulions dans le deuxième projet valider d'autres méthodes d'activation des ovocytes bovins.

De nombreuses études ont démontré qu'à la suite de la fécondation, plusieurs oscillations de l'ion calcium se produisent dans les ovocytes et que ces oscillations sont responsables du déclenchement de l'activation (Kline et Kline, 1992; Sun et al., 1992; Fissore et Robl, 1993; Shiina et al., 1993). L'incapacité des méthodes artificielles à induire des oscillations du calcium pourrait, en partie, expliquer leur inefficacité dans l'activation des ovocytes (Ozil, 1990; Vitullo et Ozil, 1992; Collas et al., 1993; Collas et al., 1995). Il faudra donc réussir à exploiter le mécanisme spermatique de déclenchement de l'activation des ovocytes non *vieillis*. Cette avenue est explorée chez différentes espèces d'amphibiens (Tompkins, 1978; Reinschmidt et al., 1979) et de poissons (Streisinger et al., 1981; Chourrout, 1982) chez qui l'irradiation permet la destruction fonctionnelle de la chromatine des spermatozoïdes sans que soit compromis leur potentiel de pénétration et d'activation des ovocytes. Il fallait vérifier si les spermatozoïdes bovins traités aux ultraviolets pouvaient toujours activer les ovocytes hôtes lors d'un transfert nucléaire. Nous avons déterminé que le taux de fécondation et d'activation des ovocytes dans le groupe ayant reçu une dose d'UV de 10 mJ/cm^2 était similaire à celui du groupe témoin alors que des doses plus élevées (30 ou 50 mJ/cm^2) inhibent la fécondation. Par contre, le développement jusqu'au stade de blastocyste a été inhibé par l'application de 10 mJ/cm^2 d'UV ce qui laisse supposer que les traitements aux UV ont endommagé la chromatine des spermatozoïdes. Cette méthode d'activation des ovocytes pourrait donc être utilisée pour le clonage des embryons.

Pour tester cette hypothèse, nous avons d'abord vérifié si les spermatozoïdes irradiés conservaient leur capacité d'activation lorsque la période de fécondation était écourtée. Il s'est avéré qu'avec une période de fécondation de 4 h, les spermatozoïdes exposés à 10 mJ/cm^2 d'UV réussissaient à activer 56 % des ovocytes, des résultats inférieurs à ceux du groupe témoin, mais néanmoins suffisants pour produire des ovocytes activés aux fins de transfert nucléaire. Les résultats ont démontré que le transfert d'un noyau diploïde n'a pas été suffisant pour renverser le blocage du développement consécutif à l'irradiation des spermatozoïdes avant la fécondation. Contrairement à ce qui est observé chez le poisson où l'irradiation aux ultraviolets peut complètement inactiver la chromatine des spermatozoïdes (Chourrout, 1984; Chourrout, 1986), il semble, d'après nos résultats, que cette méthode n'ait pas la même efficacité chez les mammifères. Nous avons finalement voulu savoir pourquoi l'irradiation des spermatozoïdes bloquait le développement embryonnaire après la fécondation.

Des ovocytes fécondés par des spermatozoïdes irradiés ont été cultivés pendant différentes périodes en présence de BrDU pour déterminer si l'irradiation affectait la réplication de l'ADN. Nous avons observé que la réplication de l'ADN se poursuivait dans tous les pronoyaux (mâle et femelle) tant dans le groupe irradié que chez les témoins. Cependant, l'incorporation du BrDU a été moins forte dans les groupes irradiés, une indication d'une réduction de la synthèse d'ADN. Hormis cette différence, il est clair que la chromatine des spermatozoïdes n'a pas été complètement détruite par les traitements aux UV.

Finalement, nous avons examiné la morphologie de la chromatine avant et après la première division et n'avons observé aucune modification morphologique des pronoyaux avant cette première division. Cependant, chez les ovocytes énucléés avant la fécondation, c'est-à-dire

qui renferment seulement le pronoyau mâle, la décondensation et la morphologie de la chromatine étaient tout à fait anormales. Il semble donc que la présence de la chromatine de l'ovocyte ait un effet bénéfique sur le remodelage de la chromatine des spermatozoïdes traités aux UV. L'effet de l'irradiation était évident chez les embryons divisés, dont la grande majorité des irradiés présentaient des altérations prononcées de la chromatine. Comme les cellules peuvent, en réponse à des agressions externes, activer des mécanismes de blocage de la division cellulaire pour éviter que des chromosomes non viables ne soient soumis à la ségrégation (Elledge, 1996), il est bien probable que les altérations induites par les UV soient responsables du blocage du développement embryonnaire. On ne peut encore expliquer pourquoi ce blocage est moins marqué chez les amphibiens et les poissons. Le plus long intervalle entre la fécondation et la division chez les mammifères permet peut-être l'activation de systèmes de réparation de la chromatine et donc des mécanismes de commande de la division cellulaire.

Dans les deux derniers volets du travail, notre objectif était d'étudier l'importance des différentes phases du cycle cellulaire sur le remodelage des noyaux transplantés. Même si plusieurs études ont déjà mis en évidence l'importance du cycle cellulaire lors de la reconstitution des embryons par transfert nucléaire (revue par Campbell et al., 1996a; Fulka, Jr. et al., 1996), son rôle dans le remodelage des composants structuraux qui commandent l'activité de la chromatine n'est pas encore élucidé. Nous avons donc concentré nos efforts sur la question de la reprogrammation de l'histone somatique H1 dans les embryons reconstitués. Cette protéine, qui se situe entre les nucléosomes et qui a des effets importants sur la morphologie et l'action de la chromatine (Clarke et al., 1998), est assemblée dans la chromatine seulement au moment de l'activation du génome embryonnaire (Clarke et al., 1992; Smith et al., 1995). La caractérisation des mécanismes de remodelage de

cette protéine pourrait donc nous renseigner sur la commande de la réorganisation structurale et fonctionnelle de la chromatine après le transfert nucléaire.

Dans la troisième partie du travail, nous voulions déterminer si parmi les modifications qui accompagnent le remodelage de la chromatine après le transfert nucléaire se trouveraient des modifications de l'histone somatique H1. Nous avons voulu vérifier si le taux plus élevé de développement obtenu avec des ovocytes hôtes conditionnés à l'aide de la méthode *télophase* par opposition à la méthode des *ovocytes vieillis* (Bordignon et Smith, 1998), se traduisait par une meilleure reprogrammation de l'histone H1. Cependant, puisque d'autres stades du cycle cellulaire des ovocytes hôtes ont un impact marqué sur l'activité de la chromatine transplantée (Barnes et al., 1993; Smith et al., 1996), nous avons comparé l'effet de cinq différents types d'ovocytes hôtes. Les résultats ont montré que l'histone somatique H1 devient non détectable immunologiquement dans la chromatine de noyaux transférés pendant le premier cycle cellulaire indépendamment du type d'ovocyte hôte utilisé. Ceci indique que les changements reliés à l'histone H1 pourraient servir de marqueurs spécifiques du remodelage de la chromatine lors du transfert nucléaire et pourrait donc être utile pour expliquer les mécanismes en jeu dans le retour à la totipotence des noyaux transplantés.

Même si les embryons reconstitués avec des ovocytes hôtes *télophase* et *vieillis* n'ont pas présenté de différences notables au regard de la cinétique des modifications de l'histone H1, le temps requis pour la perte complète de l'immunoréaction dépendait clairement du stade du cycle cellulaire des ovocytes hôtes. Les embryons induisent le remodelage de l'histone H1 bien plus rapidement quand ils ont été reconstitués avec des ovocytes hôtes avant ou peu après l'activation plutôt qu'avec des

ovocytes en interphase. La reprogrammation de la chromatine transplantée se déroulerait donc mieux quand les embryons sont reconstitués avec des ovocytes hôtes en métaphase II. Plusieurs autres études antérieures appuient cette hypothèse : des chercheurs ont pu observer la décondensation de la chromatine et la croissance des noyaux transplantés lorsque les ovocytes hôtes étaient en métaphase II, mais pas si les ovocytes avaient dépassé le stade télophase II (Czolowska et al., 1984; Szöllösi et al., 1988; Collas et Robl, 1991). Les ovocytes en métaphase II induisent de façon plus efficace l'arrêt de l'activité transcriptionnelle des noyaux transplantés (Smith et al., 1996) que les ovocytes en interphase. Finalement, le potentiel de développement à terme à la suite du transfert de noyaux provenant de cellules somatiques est supérieur quand les noyaux transplantés sont demeurés quelques heures dans des ovocytes en métaphase avant l'activation (Wakayama et al., 1998). Nos résultats en ce qui a trait au remodelage de l'histone somatique H1 suggèrent que l'effet du cycle cellulaire sur la reprogrammation des noyaux transplantés pourrait bien consister en une modification des composants structuraux de la chromatine.

Des embryons reconstitués avec les différents types de cytoplasme ont été cultivés *in vitro* jusqu'au stade de blastocyste pour vérifier si le réassemblage de l'histone somatique H1 se déroule correctement dans la chromatine des embryons en développement. Nous avons constaté que l'histone H1 réapparaît dans la chromatine chez les embryons de 8 à 16 blastomères, ce qui correspond exactement à la période où cette protéine est assemblée dans la chromatine des embryons bovins non manipulés (Smith et al., 1995). Chez les bovins, le stade de 8 à 16 blastomères recoupe la principale phase d'activation du génome embryonnaire (Telford et al., 1990). L'assemblage de cette protéine jouerait donc un rôle dans cette importante phase du développement. En effet, chez toutes les espèces étudiées jusqu'au présent, l'assemblage de l'histone somatique

H1 dans la chromatine correspond à la période d'activation du génome embryonnaire (revue par Clarke et al., 1998). Contrairement aux résultats obtenus pour le déassemblage, le stade du cycle cellulaire des ovocytes hôtes n'a pas semblé avoir un effet sur l'assemblage de l'histone somatique H1. Ces résultats indiquent qu'à tout le moins une partie des fonctions moléculaires des embryons reconstitués par transfert nucléaire est rétablie quand les embryons atteignent le stade de 8 à 16 blastomères.

Le diamètre des noyaux transplantés dans les différents types d'ovocytes hôtes a été mesuré à plusieurs reprises pendant le premier cycle cellulaire pour évaluer si les modifications de l'immunoréactivité de l'histone somatique H1 présentaient une corrélation avec la croissance nucléaire. En général, les noyaux ont subi une forte augmentation du diamètre et sont de taille similaire 16 h après le transfert sauf dans le cas des ovocytes *vieillis*, dont la croissance est quasi nulle. Selon des études menées chez les amphibiens, la croissance nucléaire est tributaire des échanges de protéines entre le noyau transplanté et le cytoplasme hôte (Merriam, 1969; DiBerardino et Hoffner, 1975). On pourrait donc en déduire que le vieillissement des ovocytes hôtes défavorise les échanges entre ces derniers et les noyaux transplantés. Cependant, on ne peut dire si cette absence de croissance a contribué au faible développement des embryons reconstitués avec les ovocytes *vieillis* comme nous l'avons observé dans des études antérieures (Bordignon et Smith, 1998). Quant aux autres types d'ovocytes hôtes évalués, même s'il semble y avoir corrélation entre la perte de l'histone et la croissance nucléaire, c'est-à-dire qu'avec le temps les noyaux augmentent de diamètre et perdent leur immunoréactivité à l'histone H1, des analyses plus détaillées semblent contredire cette hypothèse. Une nette différence peut déjà être remarquée 6 heures après le transfert : les noyaux sont approximativement de la même grandeur, mais l'histone H1 n'est plus détectée dans la majorité des noyaux transférés dans des ovocytes hôtes

en métaphase alors qu'elle est présente dans la plus grande partie des noyaux transférés dans les ovocytes en interphase. L'effet est encore plus évident 9 heures après le transfert : dans les deux groupes, les noyaux ont exactement le même diamètre, mais 100 % des noyaux transférés dans les ovocytes en métaphase sont négatifs à l'histone H1 et 100 % des ovocytes activés 6 heures avant le transfert y sont positifs. Il n'y a donc pas de corrélation directe entre la croissance des noyaux transplantés et la perte de l'immunoréactivité à l'histone somatique H1.

La dernière partie de ce projet a été consacrée à l'identification du mécanisme responsable des modifications de l'histone somatique H1 après un transfert nucléaire. Des embryons reconstitués avec des ovocytes hôtes énucléés au stade de télophase et fusionnés 2,5 h après l'activation ont été cultivés en présence d'inhibiteurs des certaines activités cellulaires comme la synthèse d'ADN (aphidilcoline), la transcription (α-amanitine), la synthèse de protéines (cycloheximide) et l'activité des protéines-kinase (6-DMAP). L'immunoanalyse pour la détection de la l'histone H1 dans la chromatine des noyaux transférés a été effectuée 12 h après la reconstitution des embryons. La culture des embryons reconstitués en présence d'aphidilcoline ou d'alpha-amanitine n'a pas affecté le temps nécessaire à la disparition complète de l'histone somatique H1. Le processus serait donc indépendant de la réplication et de la transcription de la chromatine. Ces résultats diffèrent des observations faites pendant l'assemblage de l'histone H1 dans la chromatine, qui lui est tributaire de la réplication et la transcription (Clarke et al., 1992; Smith et al., 1995). Contrairement à l'inhibition de la réplication et de la transcription, l'inhibition de la synthèse des protéines et de l'activité kinase entraîne un retard dans la perte de l'immunoréactivité de l'histone H1. Bien que cette perte d'immunoréactivité ne se manifeste pas chez tous les embryons analysés, il est clair que le processus est influencé par la synthèse de nouvelles

protéines et par l'activité de protéines-kinase même si on ne sait pas encore précisément comment. La cycloheximide pourrait, par exemple, inhiber la synthèse de l'histone H1 de type embryonnaire, qui remplace éventuellement la H1 somatique. Cependant, chez les amphibiens, la présence ou non du type embryonnaire n'a aucune importance pour le désassemblage de la chromatine de type somatique (Dimitrov et Wolffe, 1996). Par ailleurs, la cycloheximide pourrait aussi intervenir dans la synthèse des protéines responsables du remodelage de la chromatine transplantée. Même si la présence de ces protéines n'a pas encore été prouvée chez les mammifères, chez les amphibiens, le remplacement des protamines par les histones lors de la fécondation (Philpott et Leno, 1992) et le désassemblage de l'histone somatique H1 (Dimitrov et Wolffe, 1996) sont tributaires de l'action des protéines nucléoplasmines.

L'inhibition de l'activité des protéines-kinases peut aussi avoir affecté le remodelage de l'histone H1 de différentes manières. On sait, par exemple, que le MPF, principale kinase présente dans les ovocytes en métaphase, peut entre autres induire la dégradation de la membrane nucléaire et ainsi faciliter les échanges entre le noyau et le cytoplasme (Blow et Laskey, 1988). Le MPF phosphorylise aussi l'histone H1, ce qui affecte sa stabilité dans la chromatine (Hill et al., 1991). Enfin, l'activité des nucléoplasmines est aussi dépendante de la phosphorylation (Leno et al., 1996). Il serait donc possible que l'inhibition de l'activité kinase puisse affecter la perte de l'immunoréactivité de l'histone H1 par l'intermédiaire de plusieurs mécanismes.

Finalement, nous avons constaté que l'effet de l'inhibition de la synthèse des protéines et de l'activité kinase disparaissait pour ainsi dire lorsque les noyaux provenaient de blastomères issus d'une toute récente division, soit au moment où le noyau est encore au stade de G1. Le stade du cycle cellulaire des noyaux transplantés pourrait aussi être essentiel

au remodelage de la chromatine après transfert nucléaire. Cela pourrait contribuer à expliquer l'effet partiel des inhibiteurs.

La quatrième partie du travail consistait à étudier le remodelage de l'histone somatique H1 chez les embryons de souris reconstitués par transfert nucléaire. Il fallait, bien sûr, confirmer les résultats obtenus chez les embryons bovins, mais le choix de cette espèce s'explique par le fait que la commande du cycle cellulaire est beaucoup plus facile chez les embryons de souris que chez le bovin (Samaké et Smith, 1996a; Samaké et Smith, 1996b; Samaké et Smith, 1997). De plus, le taux de développement des embryons reconstitués par transfert nucléaire est moins élevé chez la souris que chez d'autres espèces de mammifères peut-être en raison de la reprogrammation incomplète de la chromatine transplantée. Ce phénomène pourrait être attribué au déclenchement de l'activité transcriptionnelle de l'embryon dès le premier cycle cellulaire chez cette espèce (Aoki et al., 1997). L'activation du génome embryonnaire s'accompagne de plusieurs modifications structurales de la chromatine (Nothias et al., 1995), dont l'acétylation des histones centrales (Adenot et al., 1997). Ainsi, chez la souris, les noyaux transplantés disposent seulement d'une courte période pour repasser par toutes les modifications qui marquent le début du développement embryonnaire. Pour mieux comprendre les mécanismes de remodelage des noyaux transplantés, nous avons voulu explorer le rôle du cycle cellulaire des ovocytes hôtes et des noyaux transplantés sur le remodelage de l'histone somatique H1.

Nous avons démontré que le cycle cellulaire des ovocytes hôtes affecte le temps nécessaire à la perte de l'immunoréactivité de l'histone H1 dans les noyaux transplantés. La perte de l'immunofluorescence a été plus rapide chez les ovocytes en métaphase que chez les ovocytes en télophase. De plus, les ovocytes hôtes utilisés 8h après l'activation sont

capables d'induire la perte de l'immunoréactivité de l'histone H1 des noyaux transplantés seulement quand ces derniers sont en métaphase. Ces résultats semblent indiquer que le faible développement des embryons de souris reconstitués avec des ovocytes en interphase (McGrath et Solter, 1984b; Tsunoda et Kato, 1997) serait consécutif à un remodelage incomplet des noyaux transplantés dans les ovocytes en interphase. Selon nos résultats, il semblerait bien que l'effet bénéfique de la présence prolongée des noyaux dans les ovocytes en métaphase sur le potentiel de développement de ces derniers (Wakayama et al., 1998) s'explique par un meilleur remodelage de la chromatine transplantée. Le taux de croissance des noyaux transplantés a aussi été influencé par le stade du cycle cellulaire des ovocytes hôtes. De la même façon que pour le remodelage de l'histone H1, la croissance nucléaire a été plus forte chez les ovocytes hôtes en métaphase et en télophase que chez les ovocytes en interphase. Cette faible croissance suggère que les échanges entre les noyaux transférés et l'ovocyte hôte seraient incomplets parce que la décondensation des noyaux transplantés est probablement dépendante des échanges protéiniques avec le cytoplasme hôte (Merriam, 1969; DiBerardino et Hoffner, 1975).

Le stade du cycle cellulaire des noyaux transplantés a aussi influencé le temps requis pour le remodelage de l'histone H1. De la même manière que pour les ovocytes hôtes, le recours à des blastomères donneurs en métaphase s'est traduit par la perte plus rapide de l'immunoréactivité de l'histone H1 par comparaison avec les blastomères en interphase. Le stade du cycle cellulaire des blastomères donneurs serait donc aussi important pour le remodelage des noyaux transplantés et le remodelage incomplet de la chromatine, la cause du faible taux de développement observé lorsque les noyaux sont transférés plusieurs heures après la division (Otaegui et al., 1994; Tsunoda et Kato, 1997).

La deuxième partie de ce projet avait pour but de déterminer si la capacité réduite des ovocytes hôtes en interphase à remodeler les noyaux transplantés était influencée par la formation du pronoyau dans l'ovocyte avant le transfert nucléaire. Nous avons montré que les ovocytes hôtes énucléés avant la formation du pronoyau induisaient un meilleur remodelage de l'histone somatique H1 et permettaient une meilleure croissance des noyaux transplantés par comparaison avec les ovocytes énucléés après la formation du pronoyau ou non énucléés. Des facteurs cytoplasmiques en jeu dans le remodelage des noyaux transplantés seraient donc assemblés dans le pronoyau des ovocytes en interphase.

Nous avons, par la suite, voulu vérifier si les mécanismes de commande du remodelage de l'histone somatique H1 étaient fonctionnels lors du transfert nucléaire entre espèces différentes. Pour ce faire, des blastomères d'embryons de souris ont été fusionnés avec des ovocytes hôtes de bovins à différentes phases du cycle cellulaire. Les résultats démontrent que le remodelage de l'histone somatique H1 se déroule à l'intérieur d'une courte période après le transfert des noyaux. Il n'existe peut-être pas de barrière entre les espèces en matière de remodelage des noyaux transplantés. Cette hypothèse est appuyée par d'autres études qui ont démontré que les ovocytes bovins fusionnés avec des noyaux de différentes espèces peuvent se développer au moins jusqu'au stade de blastocyste (Dominko et al., 1998). L'effet du cycle cellulaire des ovocytes hôtes a aussi été observé lors du transfert entre espèces : même si tous les ovocytes induisent la perte de l'immonoréactivité de l'histone H1 des noyaux transplantés, les ovocytes en métaphase le font plus rapidement.

La dernière partie de ce projet avait pour but d'identifier des mécanismes cellulaires en jeu dans le remodelage de l'histone somatique H1. Chez la souris, des embryons reconstitués ont donc été cultivés en

présence d'inhibiteurs de différentes fonctions cellulaires, soit la réplication de l'ADN, la synthèse des protéines, l'activité kinase, l'approvisionnement en énergie et l'activité des acétylases et des protéases. Bien que l'inhibition de la réplication de l'ADN et de la synthèse de protéines aient retardé le remodelage de l'histone H1 chez une partie des noyaux transplantés, aucun traitement n'a permis d'inhiber complètement la perte de l'immunoréactivité.

Ces résultats ne concordent pas tout à fait avec nos expériences antérieures chez les bovins, qui présentent une inhibition marquée de la synthèse protéinique et de l'activité kinase (Bordignon et al., 1999). L'utilisation de noyaux transplantés immédiatement après la division pourrait expliquer cette disparité puisque l'effet des inhibiteurs s'atténue grandement lorsque les noyaux sont transplantés au stade G1 (Bordignon et al., 1999).

Bien que les mécanismes moléculaires n'aient pas encore été éclaircis, l'ensemble de nos résultats démontre que le remodelage des noyaux transplantés est bel et bien influencé par le stade du cycle cellulaire lors de la reconstitution des embryons.

VII. CONCLUSION GÉNÉRALE

1. L'utilisation des ovocytes hôtes pré-activés et énucléés au stade de télophase permet d'accroître le développement et la qualité des embryons produits par transfert nucléaire.

2. Les spermatozoïdes irradiés aux ultraviolets peuvent activer les ovocytes bovins non *vieillis*, mais ils induisent l'arrêt du développement embryonnaire. Cette méthode d'activation est donc inutilisable en ce qui a trait au clonage.

3. Les ovocytes hôtes ont la capacité de remodeler des composants structuraux de la chromatine transplantée. C'est ainsi que l'immunoréactivité de l'histone somatique H1 est perdue pendant le premier cycle cellulaire pour ensuite réapparaître au stade de 8 à 16 blastomères.

4. Le stade du cycle cellulaire des ovocytes hôtes et des noyaux transplantés affecte le remodelage de l'histone somatique H1 dans la chromatine transplantée.

L'ensemble des résultats confirme l'hypothèse que le cycle cellulaire joue un rôle important dans le remodelage des noyaux transplantés.

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