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出國類別：研究 實習

草食動物人工生殖與複殖科技之研發

服務機關：行政院農業委員會畜產試驗所
彰 化 種 畜 繁 殖 場
出 國 人 職 稱：副研究員兼場長
姓 名：陳立人
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草食動物人工生殖與複殖科技之研究

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內容摘要: 本次赴法之目的爲訪視法國國家農業研究院在草食動物人工生殖與複殖科技及其他畜產生物科技之研發現況。藉由與相關研究人員討論與成果的交流，了解法國在相關畜產科技的成果與發展方向，以提供我方與法國雙方畜產生物科技研發人員進一步加強合作的著眼點和未來進一步可能合作之方向與主題的參考。同時亦對於已經在進行當中的合作計畫進行期中的討論，藉以彼此溝通並修正計畫執行的方向。另外，對於雙方都認爲很有發展潛力的生物科技相關研究課題，期望藉由原有的中法合作計畫的基礎，尋求更緊密而高度互動的合作方式。

本文電子檔已上傳至出國報告資訊網

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壹、出國目的

在「加強與法國之農業科技合作交流」計畫下，派遣行政院農業委員會畜產試驗所彰化種畜繁殖場副研究員兼場長陳立人至法國，其目的為：

1. 與法方進行動物複殖與胚幹細胞培養等相關研究的研究人員進行研習與實務上的討論，俾以提高我國家畜複殖研究的成功率。
2. 與法方研究人員討論動物複殖與胚幹細胞培養等相關研究的合作空間與模式，並商討細胞株交換等國際種原交流的可行性。
3. 與法國相關研究單位討論在我國行政院農業委員會畜產試驗所恆春分所執行的合作計畫，以俾提高計畫之執行效率。
4. 參訪法方種羊與種鵝試驗研究場，與相關研究的研究人員進行種畜禽育種繁殖等實務上的討論，以供我方畜禽育種實作之參考。
5. 加強與科技先進國家之畜產生物科技交流，提昇我國畜產生物科技水準，並促使我國相關科技研究成果的國際化。
6. 促成中法之間以細胞或細胞株的交換，做為國際間種原交流的模式。

貳、訪視過程

一、行程安排

本次出訪計畫為中法合作計畫之一，出訪地點、行程安排與研習考察內容經由在我國國立中興大學任客座教授的法國訪問學者 Roger Rouvier 博士與目前正在中法合作計劃下留學法國進修博士學位的國立中興大學陳志峰助理教授的協助規劃與安排，聯繫法國國家農業研究院（Institu National de la Recherche Agronomique, INRA）排定參訪的行程與單位。

參訪的單位包括法國國家農業研究院位於巴黎近郊的 Jouy en Josas 研究中心之生物學系與生物技術研究室（Biologie du developpment et Biotechologies），位於 Bressonvilliers 的試驗農場（Experimentral Farm of Bressonvilliers）；設立在法國中部 Tours 附近的法國國家農業研究院 Nouzilly 研究中心（Nouzilly Research Center），生理與繁殖研究場（Station de Physiologie de la Reproduction）；法國山羊選育與育種協進會（Unite nationale de selection et de promotion des races carprines），家禽研究中心（Avian Research Center），家畜生理系（Department of Animal Physiology）；設立在法國西南部地區位於 Artigueres 的水禽肥肝研究試驗場（Experimental Station of Waterfowl for Fatty Liver ）；位於 Rouille 的人工授精研究試驗場（Station Experimentale et 'd Insemination Articielle）等等。如表一所示為本次參訪的簡要行程與內容。

表一、參訪行程

日期	行程	訪視對象	訪談與討論內容
0928 to 0929, Fri. to Sat.	Taipei (23:50) to Paris (07:50)	CF Chen	Poultry breeding and genetics in INRA
0930, Sun	Paris		
1001 to 1002, Mon. to Tue.	INRA Res. Center of Jouy en Josas	Dr. Jean-Paul Renard, Xavier Vignon, Yvan Heyman.	Cattle and goat cloning
1003, Wed.	To INRA Res. Center of Tours Nouzilly by	Dr. P. Chemineau, Researchers of Station de	Goat reproductive physiology

	TGV	Physiologie animale	
1004, Thr.	Station de Recherche Avicole, Nouzilly	Dr. J.P. Brillard, Madame Nadine Sellier	Poultry genomics, photoperoids
1005, Fri.	Nouzilly, Station Experimentale et d'Insemination Artificielle(SEIA) in Rouille	Dr B.Leboeuf	Goat AI
1006-1007, Sat.-Sun.	Travel To Artigueres by TGV		
1008-1009, Mon.-Tue.	To the Experimental Station of Waterfowl for Fatty Liver in Artigueres	Mrs. Gerard Guy (Director), Jean Michel Brun	Photoperiod control in geese
1010, Wed.	Travel to Paris by TGV		
1011-1012, Thr.–Fri.	Paris (11:00) to Taipei (07:05)		

二、內容重點

（一） 在法國國家農業研究院 Jouy en Josas 研究中心之訪視與討論：

1. Jouy-en-Josas Research Center 離巴黎約 50km，約有 1000 名研究人員，分別屬於肉品加工系、生理系、遺傳系、營養系、分子發生學系及生物科技系。其附近尚有原子能研究所，其中還有數名諾貝爾獎的研究人員，在看起來不起眼的〝小村落〞裡浸淫於科學研究當中。這些研究中心的建築不是很宏偉新穎，而且每一位研究人員的辦公室也都相當地小，但是卻可以有很好的學術成績出現，這些現象值得叫人省思。
2. 在 Jouy-en-Josas 這次行程安排拜訪的主要是生物科技系專攻於牛與山羊複殖的研究團隊及從事基因轉殖的分子發生學系。

3. 牛與山羊複殖的研究團隊的主要統合人為生物科技系的主任 Jean-Paul Renard 博士，主要負責牛複殖的試驗研究者為 Xavier Vignon, 與 Yvan Heyman 博士。另外，他們針對同一個主題（Animal Cloning）集合許多不同學術領域的研究人員人去參與（包括分子生物學家、胚胎與分化學家、生理學家、病理學家），投入許多經費並從各個可能解決問題的方式去切入。這樣的團隊不但可以就現有的動物複殖所遭遇到的狀況廣泛而且深入地發掘，把造成問題的任何可能更完整地拼出來，做周沿而縝密的研究探討。相較之下，我國畜試所的動物複殖團隊則成員太少且僅限於生殖生理的學術專業，過於封閉式的組隊方式與有限的人力物力，對於相同的研究主題似乎很難與之匹敵。
4. 該團隊所產製的複殖牛有以胚細胞（桑椹期之胚葉細胞），胎兒成纖維細胞及成體成纖維細胞做為供核源者。其中 cloned 自成體成纖維細胞者，尚有來自同一頭母牛，在其不同年歲取成體成纖維細胞做複殖，以比較供核牛年歲對於牛複殖效率之影響，以目前的觀察提供核源細胞的動物年齡對於複製牛的產製、複殖牛的生長以及繁殖並沒有顯著的影響。
5. 該團隊以成體體細胞為供核源的複殖牛產製，亦見有高達 43.7%的複殖胚於懷孕階段流產損失的比率。以胚細胞為供核源的複殖牛產製，其懷孕其的損失則僅為 4.3%。胎死腹中的胎兒經剖檢，發現有各式各樣不同的器官發育不全的現象，發生的原因不明。順利懷孕產出的複殖牛亦出現有孕期延長及出生體重超重之情形。仔牛產出後的存活率亦低，約在 10%左右（如附件一、二）。
6. 該團隊對於山羊的複殖亦有高度的興趣，計劃將從 2002 年投入研究。
7. 該團隊對於本人的豬胚幹細胞研究與本人發在 1992 年表於荷蘭世界生殖學會的複殖豬成果意很有興趣。對於本人以結合豬或山羊的胚幹細胞培養與細胞核轉置建立自體細胞庫以備組織與器官移植之需的理論構想深表贊同，表示有興趣進一步合作。
8. 在 Jouy en Josas 研究中心之分子發生學系與系主任 Louis Marie Houderine 博士談論有關家畜基因轉殖的現況與發展，Houderine 博士研究 WAP 基因 promoter 已有 10 年，並擁有 WAP 基因 promoter 的專

利。他強調專利權的問題，認為台灣要發展自己的生物科技一定要投入基礎研究並且發展出自己的專利，不然以後要把研究成果商業化或技術轉移時會遭遇到很大的麻煩。

9. 在 Jouy en Josas 研究中心之分子發生學系與專攻基因表達的 Eric Pailhoux 及 P. Parma 博士討論有關性別決定基因(sex-determine genes)的研究。認為進一步研究這個相當重要而且熱門的基因之表達，與其對於家畜禽的性別控制之利用，對於未來畜產的經營會有很大的商機。

（二） 在法國國家農業研究院 Nouzilly 研究中心之訪視與討論：

1. Nouvilly 研究中心約有 1000 人，佔地約 50 公頃。其 Animal facility 有試驗動物室，負責繁殖供應包括 mice, rats, rabbits 等試驗動物。其中 mice 有 B_6、CBA 等主要做為基因轉殖研究及其他醫學研究的試驗動物。rats 主要為 Swiss 系其功能主要為醫學研究之模式動物。rabbits 不做繁殖，主要做為生產 Antibody 之用。家畜則有綿羊（3000 頭），馬（800 頭），山羊（250 頭，購自農戶淘汰者做為胚胎相關研究的材料，更新頻仍），牛（200 頭），豬（養在隔離的密閉室豬舍，約有 500 頭）。馬主要是 Pony，研究主要為體外成熟與體外受精、超音波採卵、精液冷凍製備與冷凍胚的移置等。綿羊除了胚的移置外，尚有做行為觀察之研究。牛及豬主要用在基因轉殖、胚冷凍。豬品種主要是 Yorkshire，其 30% 左右的產仔數可達 14 頭！另有純種梅山豬和少數法國土種豬（色白、肥胖、體形小，但有似 Yorkshire 之耳型，目前已瀕臨絕種）。
2. 與生殖生理系的 Pierre Orgeur 博士討論有關其正在從事的豬早期離乳之研究。研究中的豬隻在 7 日齡離乳。仔豬初期體形肚圍大而下垂，但 4 個月後和正常離乳者沒有差別。
3. 與生殖生理系的 Pascal Mermillod 博士討論有關該系在豬隻生物科技方面的研發工作。其中在豬胚冷凍方面已有仔豬出生的成果，研究係以未孵化囊胚為材料，用玻璃化法冷凍的方式進行，成功產仔的比率為 30% 。每頭受胚母豬移植 20 個冷凍胚後，懷孕率為 70-80% 。據言，胚的品種來源並不會對於冷凍的成果有重要的影響，但是受胚豬用梅山則為好方法。有關豬的基因轉殖主要是在器官移植研發方面。該研究室已擁有具人類細胞膜半乳糖基衍生物基因的基因轉殖豬品系，以及抗後發性排斥的基因轉殖豬品系，打算藉育種或基因轉殖的方式，育成兼具此二特性者（如附件三）。
4. Pascal Mermillod 博士在 Nouzilly 研究中心的團隊也打算在 2002 年開

始山羊之複殖研究。該團隊在山羊的 IVM、IVF 做得相當好。他強調 IVM 及 IVC 兩個操作階段所需之培養氣相條件不同—IVM 用 5% CO_2 in air；IVC 則必須降低 O_2 level，因此採用 5% CO_2、5% O_2 in air。P. Mermillod 擁有山羊內視鏡採山羊胚及迷你手術胚移置之技術，有必要邀請他訪指導本所的山羊生殖科技團隊，或可以安排我方相關的研究人員來此學習。

5. 與 Nouzilly 研究中心生殖生理系的 G. Baril 博士談有關 PMS 在山羊超量排卵操作之應用上所見的問題—超排成效不一，且容易引起卵巢囊腫與抗體的形成。法方也面臨相同的問題，亦曾嘗試以 PVP-FSH 代替 PMS，但是效果亦不佳。目前正在計畫進行找出最佳之超排模式。
6. 與 Nouzilly 研究中心家禽系系主任 Jean-Pierre Brillard 博士談有關該系對於家禽生物科技方面的研發。Brillard 博士是鳥類生殖科技方面的權威學者，目前專注於鳥類精子冷凍與常溫保存、精子細胞膜特性研究、家禽光照控制、以及家禽種原保存等。他認為由飼料可以改變家禽精子細胞膜脂質構成，對於其耐凍性及常溫貯存均會有影響。他也認為家禽精子在生殖道內存活的時間與遺傳有關，其機制未明，但與生殖道內分泌物之成分，尤其是 glycogen 可能有關。並謂各種鳥類之精子其代謝方式不同，因此其保存條件亦因而有明顯的差異。在與之討論家禽性腺分化的基因控制間，Brillard 博士透露這也是他目前正在收集資料打算投入研究的重要課題之一。
7. 與 Nouzilly 研究中心生殖生理系的 Benoit Malpaux 博士談有關羊的光照控制（如附件四）。他是位神經學家，利用腦下垂体廖管研究綿羊的 GnRH 釋放模式。對於 Melatonin 的亦多有涉獵。認為由日糧中增加 Melatonin 對於体內的 Melatonin 濃度並不會有顯著助益，因為體內在 Melatonin 的代謝徑路上位有 NAT 把關。在連續光照下，羊出現乏產或產季紊亂出現，是因為羊本身出現對光照刺激反應乏興奮之故。自然光照的規律變化則是提供一個 reference。讓羊群的產季可以在此提示下集中表現。而 melatonin 在非產季應用時，因為此期間內山羊對 melatonin 亦出現神經性乏興奮之現象，所以如果只給予 melatonin 而不配合光照控制，則會達不到產期調節的預期效果。之後與 Maria Pellicer 博士也是討論羊的發情同期化控制方面的問題。他自然 1966 年迄今一直在這方面做研究。因鑑於使用 PMS 上的問題，他利用光照＋公羊效應＋P_4 以解決這樣的問題，在自然交配下，受胎率為 60—65 %。以 AI 2 次方法可以達 70.3%（視品種及場別而定）。
8. 與 Nouzilly 研究中心生殖生理系專攻 neuro-stem cells 的 A. H. Duittoz

博士討論胚幹細胞與成體幹細胞的分離與培養等研究。他對於我在豬胚幹細胞方面的研究與發表深感興趣。Duittoz 博士現在正從事於由綿羊的 fetal brain 分離 neuro-stem cells，並已有初步的結果。並且可用 cytokine 有限度地指導其分化方向，可是尚有培養過程中難以控制的細胞凋亡及自主性分化無法有效抑制的問題。他的企圖是建立可以分化為控制 GnRH 分泌的 nerve cell lines。

9. 與 Nouzilly 研究中心生殖生理系的 Jean-Luc Gatti 博士談論雄性生殖生理方面的研究。這個 group 是以精子在睪丸形成後，進入附睪內如何成熟，如何獲得受精能力等變化為研究重點。探討在附睪內各部位精子的細胞膜表面分子的改變，包括從附睪液中加到精子上，以及從精子表面分泌到附睪液中的蛋白質成份，主要是以 Proteomics 的方法研究附睪液的成份為切入點，看看在不同種別（羊、山羊、牛、豬、大鼠）的成份中有何相同的蛋白質分子，並且加以氨基酸序列分析識別。在他們的研究中，以綿羊為對象時，發現精子在成熟的過程中，會將位於中片的具有 ACE 活性的蛋白質“分泌”到附睪液中。同此，從精漿中分析 ACE activity，即可反應出精液中成熟精子的數量（兩者間成正相關）。所以，可以用 ACE activity 之高低來判定此公畜的授精能力，做為公畜之品管參考。同時，此 ACE activity 之情況在牛及豬似有類似之情形（大鼠 ACE 則在頭帽，非中片）。也許，這套方法可以用在檢定公豬是否已經成熟到可以用的程度的指標，而且如果其於某年齡測量的值可以反應出其日後的表現，也許就可以更有用處—預測此公畜是否適合做為種公畜之用。另外，對於附睪液成份的分析，也許對於精液稀釋劑的改良亦有助益（如附件五）。

10. 訪視 Nouzilly 研究中心的最後是與生理系主任 Philippe Chemineau 博士對談。他與我介紹 INRA 的組織，生理系主要的工作研究組成包括：

1) 神經學與功能生物學—55 人

2) 胚胎學—95 人

3) 組織學與畜產生理學—28 人

其研究方向為：

1) 神經學與功能生物學方面：

（1） 親子識別行為學。

（2） 採食行為的分子控制機制（做為人類研究的模式）

（3） 生殖行為與排卵之神經控制。

2)胚胎發育與生物科技方面：

（1） 排卵的控制

—找出與 PMSG 及 FSH 拮抗之抗體的分子表現系統。

—發展出不用激素處理之發情同期化方法。

—研究容易及不易對激素產生抗體之動物之免疫系統對於外來物質反應之分子機制，並探討是否與抗病有關？

（2） 研究精子形成，以了解如何處理精液，以改進人工授精之效率。

—研究精液之常溫及冷凍保存。

—研究以光照控制公羊做為提供人工授精精液之來源。

—在馬、羊、豬及綿羊精液保存方面，尋求延長使用時間及用量少且高受精率之保存方式。

（3） 排卵時間與卵品質提昇之研究

—利用光照＋公羊效應＋P_4以替代 PMSG。

—提升激素的效率以提高超量排卵的效果。

—濾泡形成之分子機制之探討。

（4） 體外成熟與體外受精

—找出與影響後體外成熟與體外受精之卵發育成為囊胚之主要影響因素，以提高體外成熟與體外受精之效率。

—找出與以成體細胞做複殖時，發育成為囊胚之主要影響因素，以及影響複殖胚 reprogramming 成功之基因因素。

（5）基因轉殖研究

—建立人類醫學之遺傳研究動物模式。

Philippe Chemineau 博士指出，生理系本身與其他 INRA 各個研究中心、附近的大學院校、其他研究機構、私人生技公司及國外歐盟研究單位都有很好的合作關係。也希望透過中法合作，提出可行而彼此都有興趣，而且有貢獻相當的計畫，進行實質的合作。

他指出，這是科學家的合作，是沒有任何其他包括政治考量的；我也說 "Cooperation in Science is to bring brains and ideas together but no political manner." 他也深表讚同。雙方表示在我把此行所見所聞帶回去，並且讓所內甚至國內的研究人員廣為週知，再提出可行且雙方都有興趣及能力執行、而且可以有彼此貢獻相當的計畫，尋求透過中法共同合作進行。

（三）在法國國家農業研究院水禽肥肝研究試驗場之訪視與討論：

1. 場長 Gerard Guy 介紹水禽肥肝研究試驗場之研究方向包括：

1) 動物福利

（1） 強制餵食與緊迫

（2） 鴨與鵝對強制餵食的行為反應

（3） 鴨與鵝品種與強制餵食的關係

（4） 強制餵食的痛覺反射

2) 環境與營養

（1） 糞便中有機磷排出量的減少

3) 肉質之相關研究

（1） 營養與肉質的關係

（2） 肉質成份分析與數據化研究

4) 生殖生理與育種

（1） 郎德鵝品系選育

（2） 光照調節鵝產期之研究

（3） 鵝精液冷凍

（4） 鵝人工授精

（5） 鴨授精持續期之探討

（6） 鴨胚早期死亡之探討

（7） 鴨人工授精與繁殖管理

2. 與 Jean-Michel Brun 博士討論郎德鵝品系選育以及利用光照控制調整產期方面的研究。郎德鵝經過選育後，在產蛋數提高與產蛋期的延長方面均有改善的趨勢，但是效果上未有顯著性。在利用光照控制調整產期方面的研究方面，以人工光照控制下，種鵝的產蛋數由 38 個提高到 69 個。與本場的種鵝光照試驗有相類似的結果。然其對於鵝隻對於光照之生理反應機制並未有深入的探討。

（四）在法國國家農業研究院人工授精研究試驗場之訪視與討論：

1. 此場位於 Rouille 是以豬和山羊人工受精相關科技的研發與推廣而設立的研究試驗場。場裡除了生產豬、羊的新鮮與冷凍精液以供農民之需外，亦於 2000 年起開始從事胚冷凍的研究。

2. 與參與中法合作計畫山羊產季調節的 Bernard Leboeuf 討論計畫的進程。他對於恆春分所的計畫執行建議：

1) 注意供試羊隻年齡的選擇，應以 7 到 11 月齡者為佳。

2) PMS 的用量分每頭劑量為 250 iu 與 330iu 兩組進行測試，注射的容量為 1 到 2ml。

3) 於 PMS 注射後第七天利用超音波掃描或內視鏡觀察排卵情形，並密切觀察發情。

4) 泌乳羊的 PMS 用量則以泌乳量做標準，超過 3.5kg 者每頭給予 500iu，泌乳量在 3.5kg 者以下者每頭給予 400iu。

5) 母羊分娩後授精的時間，以分娩後 5 到 8 週為佳；並且在觀察到發

情後 20 到 24 小時進行人工授精為宜。

參、心得

一、法國國家農業研究院有關畜產研究試驗的研究人員編制龐大，針對單一重要的研究課題不但有各相關領域的研究人員從各方面、不同角度去深切投入，呈現極細密而且既深又廣的專業分工。再配合上支援人力、研究經費與時間的投入，對於研究主題的宏觀與微觀均有全面性的涵蓋。畜產研究在法國得國家與民間都受到很高度的重視與支持。

二、法國國家農業研究院各研究中心與試驗場與大學院校、其他研究機構、私人生技公司及國外歐盟研究單位都有很好的合作關係。彼此之間的互動頻繁，產官學界人員的相互交流和支援已為常態，對於科技的研發、推廣與技術轉移有很大的助益。

三、本次訪視拜會的各個法國國家農業研究院單位在畜產生物科技的研發方面的成果與進程，都已臻達國際一流的水準。由其在草食動物人工生殖與複殖科技方面，已有龐大的團隊依循縝密的計畫正在推動。其團隊的組成與面對問題的計畫統合和整編方式，實很值得我國學術界加以借鏡。

四、法國國家農業研究院對於研究成果所衍生的專利權十分重視，這不但是保護研究人員的智慧財產，也是國家競爭力的指標，更是生物科技的研發是否得以生根發展、技術轉移是否可以為國家帶來利益的重要關鍵。

五、本次訪視拜會的各個法國國家農業研究院單位對於與我方相關的研究主題，尤其是草食動物人工生殖與複殖科技以及胚幹細胞的研究表現很高度的興趣與合作的意願。值得進一步派員討論相關的合作與交流研究人員諸等事宜。

六、法國對於鵝隻的品系選育雖然已經投入多年，但是成效相當有限。這樣的結果與我國行政院農業委員會畜產試驗所彰化種畜繁殖場的研究成果相似。但法方認為品系選育是必須長期投入的工作，目前仍持續進行。也期待我國在這方面持續努力，並且借著中法合作計畫分享經驗與成果。在種鵝光照控制產期研究方面，我國與法方幾乎同時將研究室的試驗成果推到現場去驗證。法國國家農業研究院的相關單位對於這樣相似的進境相當有興趣，期望在各自努力之後，可以將此推展的結果和經驗更實質而深入地彼此分享。

肆、建議

一、 在研究人員的互訪與出席國際會議方面，建議在經費與人員數量上多予大力支持。一方面可以增進彼此在相關課題上的討論機會，讓我國的研究人員了解國際研發現況，另一方面也可以增加國際學術研究界對我國科技研發的進程和發展方向之了解，以提高國際研究的合作機會與我國研究成果的國際能見度。

二、 在國際研究人員的交流方面，建議多提供外國專家來台的長期參與國內研究的機會以引進國際水準的科技。另一方面也建議多派遣國內的研究人員駐外參與國外高水準的研究，以提升我國畜產科技的研發能力，進一步提高我國的學術研究水平。

三、 中國有計畫地派遣在法國國家農業研究院長期接受再訓練與學術交流的研究人員相當地多，而且補助的待遇亦相當地優渥。其參與的研究，尤其在畜產生物科技方面，亦相當地深入與紮實。相較之下，我們與法國的中法合作計畫就相當地薄弱。而法國在畜產生物科技方面的研發已達國際第一流的水準，且具有很高的潛力與競爭力。職是之故，建議我國應該對於這樣的態勢加以更高度的重視，在舊有的中法合作計畫的基礎上，重新思考並構築更綿密且有利於我國生物科技發展的合作方向，以提高中法合作計畫的深度與廣度，進一步提高我國未來生物科技研究發展的國際競爭力。

四、 法國國家農業研究院的草食動物人工生殖與複殖科技團隊對於本次訪視人有關胚幹細胞的研究成果與家畜禽性別控制具有高度的興趣，希望進一步在中法合作計畫下針對這二方面的研究有更密切而深入的合作。建議透過相關研究人員的交流以對這個具有高度發展潛力的課題做更深切而完整的探討。

五、 生物科技研發成果或技術專利等的智慧財產權宣告與保護，將決定國家生物科技紮根與發展之重要關鍵。建議我國農政單位設立專責機構收集分析國際相關專利資訊，並協助學術研究單位處理其研發成果的智慧財產權保護等相關事務。

六、 官方機構與民間生物科技公司間研究人員的交流，乃是帶動與指導生物科技產業發展的重要動力。然我國在這方面卻有多種限制。期望我國可以建立一套合理完善的制度，讓產、官、學界的合作關係，尤其是研究人員的交流方面，可以在平等互惠的基礎上，更加暢通與活絡。

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Frequency and Occurrence of Late-Gestation Losses from Cattle Cloned Embryos

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ABSTRACT

Nuclear transfer from somatic cells still has limited efficiency in terms of live calves born due to high fetal loss after transfer. In this study, we addressed the type of donor cells used for cloning in in vivo development. We used a combination of repeated ultrasonography and maternal pregnancy serum protein (PSP60) assays to monitor the evolution of pregnancy after somatic cloning in order to detect the occurrence of late-gestation losses and their frequency, compared with embryo cloning or in vitro fertilization (IVF). Incidence of loss between Day 90 of gestation and calving was 43.7% for adult somatic clones and 33.3% for fetal somatic clones, compared with 4.3% after embryo cloning and 0% in the control IVF group. Using PSP60 levels in maternal blood as a criterion for placental function, we observed that after somatic cloning, recipients that lost their pregnancy before Day 100 showed significantly higher PSP60 levels by Day 50 than those that maintained pregnancy (7.77 ± 3.3 ng/ml vs. 2.45 ± 0.27 ng/ml for normal pregnancies, $P < 0.05$). At later stages of gestation, between 4 mo and calving, mean PSP60 concentrations were significantly increased in pathologic pregnancy after somatic cloning compared with other groups ($P < 0.05$ by Day 150, $P < 0.001$ by Day 180, and $P < 0.01$ by Day 210). In those situations, and confirmed by ultrasonographic measurements, recipients developed severe hydroallantois together with larger placentome size. Our findings suggest that assessing placental development with PSP60 and ultrasonography will lead to better care of recipient animals in bovine somatic cloning.

assisted reproductive technology, conceptus, embryo, placenta, pregnancy

INTRODUCTION

Successful somatic cloning has now been achieved in several mammalian species as reported by the birth of offspring in sheep, cattle, goats, pigs, and mice [1–9]. The overall efficiency of the technique, however, remains low because only a very limited percentage (0.5%–5%) of the reconstructed embryos result in full-term development. This is mainly due to a high frequency of postimplantation developmental arrest, which can occur after the transfer of blastocysts that appear to be morphologically normal. Such a situation is clearly demonstrated in mice [8, 9] or in cattle [10, 11]. Recent reports on cattle [12, 13] show that fetal losses associated with placental abnormalities are predominant during the first trimester of the 9-mo pregnancy in this species, but can occur much later, which is very unusual under natural reproduction conditions.

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These long-lasting effects of cloning are associated with excessive accumulation of allantoic fluid and increased fetal or birth weight [4, 13, 14]. This syndrome is similar to large offspring syndrome (LOS), which has been reported previously for in vitro-derived blastocysts in sheep and cattle [15, 16]. LOS is related to in vitro culture conditions of embryos before their transfer to recipient females [17] and/or to adverse effects associated with exposure of normally grown embryos to an advanced uterine environment [18]. This raises the question of whether the high incidence of late gestation losses in pregnancy after cloning is mainly due to in vitro culture conditions or to associated reprogramming effects of the reconstructed embryos by nuclear transfer.

Several cases of pathologic placentation during pregnancy after somatic cloning have been described. Transfer of blastocysts derived from adult mural granulosa cells by Wells et al. [4] has resulted in the loss of fetuses in the third trimester of pregnancy, due to an excessive accumulation of allantoic fluid. In a preliminary report, we observed three cases of recipients developing hydroallantois (grossly abnormal abdominal distension) from 6 mo to the end of gestation out of 20 recipients that carried cloned embryos that were derived from fetal or adult fibroblasts [13]. However, we had previously observed that the incidence of late abortion was relatively low (10%) in our laboratory after embryonic cloning, and that LOS was limited to 3% of the calves born [19]. We have now extended these preliminary observations by comparing the incidence of late-gestational effects resulting from the transfer of embryonic or somatic cloned embryos cultured up to the blastocyst stage under similar conditions.

To monitor the evolution of pregnancies up to parturition, we combined different methods to check placental and fetal normality or abnormality. Such information is important because the different organs of the fetus are already formed by that time and should be growing, as was described many years ago by bovine embryologists [20, 21]. Repeated ultrasonography has been used to evaluate fetal growth in horses from 100 days of gestation to parturition [22], but this technique has to be adapted to the bovine species, in which visualization of the fetus is more difficult by the rectal route after 3 mo of pregnancy. Placental development can also be evaluated by maternal levels of pregnancy proteins such as pregnancy-specific protein B [23], pregnancy-associated glycoprotein [24], and pregnancy serum protein 60 (PSP60), a protein of 60 kDa [25]. Except for some biochemical differences, these proteins are similar. PSP60, which is secreted by the binucleated cells of the placenta, is a specific marker of pregnancy in cattle and is easily assayed from a blood sample of the dam [25].

Late losses have a serious economic effect on the cost of generating cloned offspring because recipients have to be kept under controlled conditions for several months

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without a final result. They also expose the recipients to conditions that threaten their welfare. The possibility of predicting the occurrence of a pathologic evolution of pregnancy, by combined use of ultrasonography and pregnancy-specific protein assays on the recipient, would be very useful for the better care and management of the recipients in order to improve their welfare. We therefore examined the possibility of using PSP60 measurements as an early predictor of late gestation failure.

The objectives of this study were 1) to follow the evolution of pregnancy after somatic cloning in order to detect the occurrence of fetal loss and its frequency compared with embryo cloning or IVF and 2) to establish accurate criteria for predicting abnormal fetal or placental growth during late pregnancy.

MATERIALS AND METHODS

Embryo Production

All the blastocysts transferred to recipients were produced in vitro through embryo or somatic nuclear transfer; control embryos were derived through IVF.

Oocyte preparation. Bovine ovaries were collected from two different abattoirs, washed several times with fresh saline solution, and then transported in sterile PBS at 33°C to the laboratory within 3 h of collection. Cumulus oocyte complexes (COCs) were aspirated from follicles 2–6 mm in diameter, washed in HEPES-buffered TCM-199, and selected on the basis of their morphology for in vitro maturation according to the density of their cumulus cell layers. For in vitro maturation, groups of 30–40 COCs were incubated in TCM 199 supplemented with 10% (v/v) fetal calf serum, 10 μg FSH ml^{-1}, and 1 μg LH ml^{-1} for 22 h at 39°C in a humidified atmosphere of 5% CO_2 and air. At the end of the maturation period, oocytes from the same batch were used either to provide a recipient cytoplast for cloning or for IVF (i.e., the IVF embryo control group).

Embryo nuclear transfer. Embryo cloning was performed as previously described by our laboratory [26]. Briefly, in vitro-matured oocytes had all their cumulus cells removed by gentle pipetting after exposure to TCM 199 supplemented with 0.5% (w/v) hyaluronidase (Sigma Chemical Co., St. Louis, MO) for 10 min. Oocytes were incubated in TCM 199 supplemented with 0.5 μg Hoechst 33342 ml^{-1} (bisbenzimide, Sigma) for 20 min in order to stain chromatin, and were then enucleated by micromanipulation under an inverted microscope (Olympus IMT2) equipped with epifluorescence and an intensified camera (LHESA, France). The efficiency of enucleation was directly checked by controlling the presence of the metaphase plate and polar body in the enucleation pipette. For embryo cloning, cytoplasts were preactivated by aging and cooling prior to fusion with blastomeres isolated from Day 6 in vivo-produced or in vitro-produced morulae of the Holstein breed. Fusion was achieved by electrostimulation (Grass stimulator, 1.3 kV for 50 μsec in 0.3 M mannitol solution) [27].

Somatic nuclear transfer. For somatic cloning, donor cells were cultured over several passages (3 to 12) from fetal or adult skin biopsies processed as previously described [3] on five genotypes. The fibroblasts were grown in 60-mm Petri dishes in order to obtain either a growing or a quiescent population of cells on the day of nuclear transfer. Just before nuclear transfer, the cells were mechanically scraped, pelleted at 1200 × *g* for 5 min, and resuspended in fresh TCM 199. Each isolated cell was inserted under the zona pellucida of the recipient cytoplast and fused by electrostimulation [3].

In vitro development of nuclear transfer embryos. All the reconstituted embryos from embryonic or somatic cells were cultured under the same conditions (i.e., in microdrops of 50 μl B2 medium [CCD, Paris, France] supplemented with 2.5% fetal calf serum and seeded with Vero cells according to the culture system used in our laboratory for bovine IVF embryos) [28]. The droplets were overlaid with mineral oil (M8410 Sigma Co., NY) and incubated for 7 days at 39°C under 5% CO_2. Cleavage was assessed at Day 2 after fusion and blastocyst formation was evaluated at Day 7. Expanding or early hatching blastocysts (grades 1 and 2) by Day 7 were removed from the culture drops and used for transfer to recipient heifers.

Control IVF embryos. Control groups of IVF embryos were obtained from the same batches of in vitro-matured oocytes. Twenty-four hours after onset of maturation, oocytes were coincubated with heparin-capacitated frozen-thawed spermatozoa in Tyrode albumin lactate pyruvate medium for 18 h according to the standard technique routinely used in the laboratory [29]. A single batch of frozen sperm from the same Holstein bull was used throughout the experiment to produce the control embryos. After IVF, presumptive zygotes were cultured until Day 7 under the same conditions as nuclear transfer embryos. By Day 7, blastocysts of grades 1 and 2 quality were used for transfer to recipients.

Embryo Transfer

Animals. Recipient animals were beef-breed Charolais or cross-bred heifers raised under the same conditions and transported to the experimental farm by the age of 12–14 mo. They were certified free of all major infectious cattle diseases by repeated serological testing prior to transport. They were then checked for normal cyclicity before being used as recipients for embryo transfer by the age of 15–18 mo.

Estrous cycles were synchronized in each group of recipients using a progestagen implant for 9 days (Crestar Intervet) associated with a prostaglandin analogue injection (2 ml Estrumate) 2 days before implant removal. After estrus detection, heifers that were synchronous ± 24 h with embryo age and carrying a palpable corpus luteum were selected for embryo transfer.

Day 7 blastocysts that were developed in vitro after nuclear transfer or IVF had the same age at time of transfer (Day 0 being the time of fertilization or nuclear transfer). They were loaded into 0.25-ml straws (IMV, L'Aigle, France), one embryo per straw, and transported in a thermos flask at 39°C from the laboratory to the experimental farm. Embryo transfer was performed nonsurgically into the uterine horn ipsilateral to the corpus luteum (single transfer) using the miniaturized embryo transfer syringe and sheath (IMV) under slight epidural anesthesia.

Pregnancy Monitoring

Plasma progesterone assay. A heparinized blood sample was taken at Day 21 (2 wk after transfer) from each recipient by venipuncture of the caudal vein and centrifuged immediately. Plasma was separated and frozen before being assayed for a rapid estimation of progesterone concentration by radioimmunoassay. Recipients were considered nonpregnant if progesterone concentration by Day 21 was <1 ng/ml and presumed pregnant when concentration was >2 ng/ml.

PSP60 assay. Concentrations of PSP60 were measured in the peripheral blood of recipients from frozen plasma samples stored after monthly blood venipuncture by radioimmunoassay as described by Mialon et al. [25]. Sensitivity of the PSP60 assay was 0.2 ng/ml plasma, and intraassay and interassay coefficients of variation were 6% and 12%, respectively. The cutoff minimum/maximum levels were 0.2 and 6.2 ng/ml. For higher concentrations in late gestation, plasma samples were diluted accordingly. The PSP60 levels in recipients that became pregnant from embryonic or somatic clones were compared with those of a group of control animals that became pregnant after artificial insemination in order to evaluate whether this measurement can be a predictive criterion for detecting abnormal development of placenta in cloned fetuses.

Ultrasonography. All recipients were examined for the presence or absence of a viable fetus at Day 35 ± 2 days using transrectal ultrasonography (Pie medical ultrasound equipped with a 5.0 MHz probe). Pregnant recipients were then repeatedly checked on Days 50, 70, and 90 of pregnancy.

From Day 120 of pregnancy, recipients carrying a somatic clone fetus or a control fetus were submitted to repeated transabdominal ultrasonography using a 3.5 MHz probe, every 2 wk until calving. The viability of the fetus as well as the ultrasonographic aspect of the placenta were thus monitored. Contemporary pregnant heifers (through artificial insemination) of the same breed were used as controls and to establish a normal range (n = 13). Fetal heartbeat was recorded and the aortic diameter was measured just outside the heart as described in other species [30, 31] as a possible criterion for oversize. For placental evaluation, the size of placentomes was estimated by measuring the surface on the screen at their maximal size. We measured in each recipient the size of four placentomes localized in the same ventral area close to the udder. Because no reference values exist, we established these reference values on control animals at the same stages of gestation. Qualitative aspects (visualization or absence of edema estimated by contrast levels of tissues on the screen) were also recorded.

Transabdominal ultrasonography was performed in order to detect any pathologic evolution of pregnancy such as abnormal fetal growth (aortic diameter), fetal stress, or hypoxia (heartbeat). Placental abnormalities or severe hydroallantois was detected by an increase in fluids associated with difficulty in locating the fetus in the uterine cavity. It was accompanied

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TABLE 1. In vivo development after transfer of cattle cloned embryos.

	Group 1, somatic adult	Group 2, somatic fetal	Group 3, embryonic	Group 4, control IVF embryos
No. recipients	133	40	67	51
Presumed pregnant Day 21	74/133	23/40	42/67	32/51
	55.6%[a]	57.5%[a]	62.6%[a]	62.7%[a]
Confirmed pregnant Day 35	45/133	11/40	33/67	27/51
	33.8%[a]	27.5%[a]	49.2%[b]	52.9%[b]
Pregnant Day 50	36/133	9/40	28/67	26/51
	27.1%[a]	22.5%[a]	41.8%[b]	50.9%[b]
Pregnancies by Day 70	19/133	9/40	25/67	25/51
	14.3%[a]	22.5%[a,b]	37.3%[b,c]	49.0%[c]
Pregnancies by Day 90	16/133	9/40	23/67	24/51
	12.0%[a]	22.5%[a,b]	34.3%[b,c]	47.0%[c]
No. calves born	9/133	6/40	23/67*	25/51*
	6.8%[a]	15.0%[a]	34.3%[b]	49.0%[b]

* Including one twin calving from single embryo transfer.
[a-c] Within the same line, percentages with different superscripts are significantly different, $P < 0.05$ or 0.01).

by a progressive deterioration of the clinical status of the recipient (loss of appetite, body condition). When several criteria from at least two successive ultrasound examinations confirmed that the pregnancy was abnormal, the pregnant recipient was humanely killed before her clinical status became critical, and the uterus and the fetus were recovered for examination.

Calving

Pregnant recipients were kept until calving. Cloned calves were delivered by cesarean delivery following maternal treatment with 20 mg dexamethasone (Dexadreson) i.m., 36 h before surgery, when natural calving had not occurred by Day 282 of pregnancy. Calving was scored according to the ease of delivery (from 1 = no assistance, to 4 = cesarean delivery). Newborn calves were given colostrum within 2 h after calving, and birth weight, sex, and the length of gestation were recorded for each group of in vitro-produced embryos. LOS was considered to occur when birth weight was higher than the mean birth weight plus two standard deviations within the same herd [31]. Calves were isolated and followed up to the age of 2 mo.

Experimental Design and Statistical Analysis

Evolution of pregnancy rates was followed on four groups of recipient animals over a 3-yr period. For each group, at least part of the transfers were contemporary with the other groups:

Group 1 = recipients of adult somatic cloned embryos (n = 133).
Group 2 = recipients of fetal somatic cloned embryos (n = 40).
Group 3 = recipients of embryonic cloned blastocysts (n = 67).
Group 4 = control group, recipients of IVF embryos (n = 51).

Pregnancy rates and calving data were compared for the four groups using ANOVA.

Placentome size, fetal heartbeat, and aortic diameter were measured by ultrasound, comparing pregnant recipients in groups 1 and 2 (somatic cloning, n = 21) and normal pregnancies in a control group of animals that became pregnant through artificial insemination (n = 13). For each gestational period, the Student *t*-test was used.

PSP60 assays were performed on some of the recipients in groups 1 and 2 (somatic cloning, n = 30) and group 3 (embryo cloning, n = 22). For each stage of pregnancy, values were averaged for recipients that developed pathologic pregnancies after somatic cloning. Reference values for PSP60 levels in normal pregnancies were obtained from a monthly sampling of a group of 11 contemporary cows that became pregnant through artificial insemination.

RESULTS

Evolution of Pregnancy after Transfer of Cloned or Control IVF Embryos

Pregnancy results for the four groups of recipients are presented in Table 1. Transfer of IVF embryos, embryo clones, and fetal or adult somatic clones resulted in the same range of initiated pregnancy rates (55.6%–62.7%). However, evolution of pregnancy rates was quite different between groups. Confirmed pregnancy rate by Day 35 assessed by ultrasonic scanning was significantly lower in group 1 (somatic adult, 33.8%) and group 2 (somatic fetal, 27.5%) compared with the controls, group 4 (IVF; 52.9%; $P < 0.01$). At Days 50 to 90, pregnancy rates were confirmed to be significantly lower in groups 1 and 2 than in groups 3 or 4 ($P < 0.05$).

Placental and fetal development in pregnant recipients after somatic cloning was monitored using repeated scanning on the same animals every 2 wk from the fourth month of pregnancy until calving, in order to detect any pathologic evolution. Placentome sizes were recorded from 21 recipients that were pregnant from somatic clones, and compared with those of 13 control animals (artificial insemination). Results presented in Figure 1 indicate that in recipients that carried somatic clones, the mean size of placentomes between 4 and 6 mo was significantly higher than in control pregnancies, regardless of the outcome of pregnancy. There was a linear regression between placentome size and stage of pregnancy in clones and controls ($r^2 = 0.84$ for clones, $P < 0.01$; and $r^2 = 0.69$ for controls, $P < 0.05$).

Fetal heartbeat was recorded every 2 wk in clones and control fetuses. No significant differences were observed. Whatever the pregnancy stage, frequency of heartbeat was >100 per min. There was a slight tendency toward decreasing frequency by the end of pregnancy. By Day 262, heartbeat was 116 ± 11.9 per min, and 100.5 ± 14.8 per min for somatic clone and control fetuses, respectively. Scanning was also used to measure the aortic diameter in clone and control fetuses. Evolution of aortic diameter was not significantly different between clone and control fetuses. There was, however, also a significant correlation between gestational age and aortic diameter in clones ($r^2 = 0.60$) and in controls ($r^2 = 0.62$).

Frequency of Late Fetal Losses

The proportion of late-gestation losses between Day 90 and calving reached 43.7% in recipients carrying adult somatic clones and 33.3% for fetal somatic clones, whereas only 4.3% of the pregnancies were lost during the same period after embryo cloning, and no late loss was detected in the control IVF group (Table 2).

Using repeated scanning on 21 recipients carrying somatic clone fetuses, 5 cases of late abnormal pregnancies were detected, and recipients were killed between Days 155

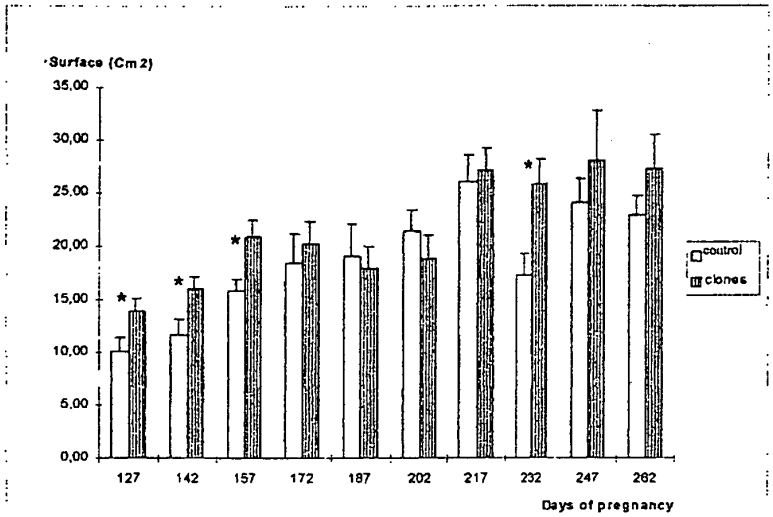

FIG. 1. Evolution of placentome size during pregnancy after somatic cloning. X-axis, stage of pregnancy (days); Y-axis, mean size of placentomes (cm² ± SEM). *Significantly different from controls ($P < 0.05$).

and 233 of gestation. In both cases severe hydroallantois was confirmed at autopsy and the size of placentomes was measured after dissection of the uterus. Mean placentome weight was 142.3 ± 61.7 g compared with 46.7 ± 22.7 g for placentomes in three cases of normal pregnancy at the same stage. Severe hydroallantois was often associated with fetal abnormalities (Table 3); three fetuses presented LOS and one was completely hydropic.

PSP60 Profiles and Late Losses

PSP60 concentrations were measured on blood samples from recipients between Day 35 and calving. During the first trimester of pregnancy (Fig. 2, a and b), PSP60 concentrations increased from Day 35 to Day 90 in recipients that were confirmed pregnant at 3 months and, for each stage of pregnancy (Days 50, 70, or 90) there were no significant differences in the mean levels of PSP60 concentrations between groups that further developed to full-term pregnancy. However, for recipients of the somatic clone group, which subsequently lost their pregnancy as detected by ultrasound scanning (Fig, 2a), we found a significantly higher level of PSP60 by Day 50 than for those that maintained pregnancy (7.77 ± 3.3 ng/ml vs. 2.45 ± 0.27 ng/ml for normal pregnancies, $P < 0.05$). These higher levels were detected at Day 50 even though pregnancies were lost before Day 100 in these animals. Moreover, PSP60 levels over 4 mo of pregnancy appeared to be significantly increased in animals in which pathologic pregnancy was detected by ultrasonography in late gestation (Fig. 2c). This was true only for somatic cloning compared with other groups ($P < 0.05$ by Day 150, $P < 0.001$ by Day 180, and $P < 0.01$ by Day 210). In those situations, the plasma PSP60 concentrations in recipients that developed severe hydroallantois were very high, up to 400 ng/ml by Day 180 of pregnancy, when levels in other groups were less than 100 ng/ml at the same stage. No significant difference was observed in the PSP60 concentrations in recipients that delivered live, full-term calves after embryo or somatic cloning compared with the control group.

Calving and Postnatal Survival

Proportions of calves born differed significantly between groups (6.8% in group 1 [adult somatic cloning], 15.0% in group 2 [fetal somatic cloning], and 34.3% in group 3 [embryo cloning], respectively, compared with 49.0% in the controls, group 4 [IVF], $P < 0.01$).

Information on calves born, birth weight, length of gestation, and postnatal survival for the different groups are given in Table 4. Mean birth weight of adult somatic cloned calves was significantly higher than that of control IVF calves (53.1 ± 2.0 kg compared with 44.5 ± 2.1 kg for IVF calves, $P < 0.05$). Calving score was higher for recipients carrying somatic clones due to the high proportion of cesarean deliveries performed in these groups. Postnatal

TABLE 2. Proportion of late losses in the different groups of recipients.

Group	No. pregnancies (Day 90)	Deaths at 3–5 months	Deaths at 5–7 months	Deaths at 7–9 months	Total late loss
Adult somatic clones	16	3/16	3/16	1/16	7/16
		18.7%	18.7%	6.2%	43.7%
Fetal somatic clones	9	0/9	2/9	1/9	9/9
		0%	22.2%	11.1%	33.3%
Embryonic clones	23	0/23	1/23	0/23	1/23
		0%	4.3%	0%	4.3%
Control IVF	24	0/24	0/24	0/24	0/24
		0%	0%	0%	0%

TABLE 3. Characteristics of somatic clone fetuses recovered at slaughter from recipients developing abnormal pregnancies.

Fetus number	Age at recovery (days)	Weight (kg)	Crown-rump length (cm)	Gross pathology of the fetus at necropsy	Placenta
1 (B431)	215	24.6	85	LOS + excess of peritoneal fluid	hydroallantois enlarged placentomes
2 (B386)	233	84.0	105	LOS + bulldog calf syndrome	hydroallantois
3 (B383)	184	16.0	63	abnormal kidneys, hydropic	hydroallantois
4 (B417)	202	22.4	80	LOS, enlarged umbilicus	hydroallantois
5 (B456)	155	3.5	40	Hypertrophic liver (7% of body weight)	hydroallantois (aborted)

survival of live-born calves was satisfactory (>80% survival after 1 mo) for three groups (control IVF, embryo clones, and somatic fetal clones). Calves born from adult somatic cloning had a lower survival rate; 33% (3 of 9) died during the first week but 6 of 9 were alive after 1 mo and developed normally.

DISCUSSION

In the present study, we describe the evolution of pregnancies after somatic cloning and report the high incidence of late-gestation losses. These losses occurred later and at a higher rate than after embryonic cloning. Combined use of repeated scanning on the same animals throughout pregnancy and PSP60 assays allowed us to monitor placental and fetal development, and to detect the occurrence of losses as well as development of pathologic pregnancies in the groups of recipients carrying fetal or adult somatic clones. This was not the case in the control group and, to some extent, in the embryo clone group, in which the pregnancies diagnosed at 3 mo were all maintained, except one (in the embryo group), until calving. Because all the embryos were cultured under the same in vitro conditions, the higher incidence of late-gestation losses after somatic cloning compared with embryo nuclear transfer or IVF, reflected the consequence of some defective reprogramming process [32] and not a deleterious effect of culture. We can exclude uterine overcrowding as a possible cause of loss in these experiments, because only one embryo was transferred per recipient, whereas this is not the case when more than two embryos are transferred, as reported by Hill et al [12].

Maternal secretion of pregnancy-specific proteins is generally used as a simple pregnancy test in cattle [23, 24]. Our objective in measuring PSP60 levels was also to try to find a predictive criterion for normal/abnormal pregnancy. We observed that an abnormal gestation outcome in recipients that carried embryonic or somatic clones was linked to high levels of PSP60 in the blood. The PSP60 protein is secreted by the binucleated cells in the fetal cotyledons and can be detected in maternal blood. The circulating level of pregnancy-specific protein has been used as a criterion to discriminate between single and twin pregnancies after artificial insemination or embryo transfer in cattle [33]. The levels of PSP60, if significantly increased, could be a good indicator of abnormal placenta development in some of the recipients that carried somatic clones. This was observed as early as Day 50 in recipients that later lost their fetus after somatic cloning, as the level of PSP60 was significantly increased in such recipients. Hill et al. [12] reported that pregnancy-specific protein b was higher at Day 35 in the pregnancies that failed by Day 90. By Days 150 and 180 of pregnancy, mean levels of PSP60 were also significantly higher in recipients that carried somatic clones and developed pathologic pregnancies, which suggests an overactive placental secretion of this glycoprotein. This was further confirmed by scanning measurements of placentome sizes, which were significantly larger in the five recipients of this somatic clone group that clearly developed hydroallantois. Autopsy of these recipients after slaughter confirmed the occurrence of this placental pathology in all cases. Postmortem dissection of the recipient uterus showed the presence of large-size hydropic cotyledons, some of which weighed up to 0.5 kg. This observation is in agreement with the high level of PSP60 detected. The fact that recipients that developed pathological pregnancy showed an increase in PSP60 levels about 1 mo earlier than recipients that carried normal pregnancies can be used to detect an overactive placenta. This is in accordance with recent observations by Farin et al. [34], who indicated that at 63 days following embryo transfer, bovine placentas from embryos produced in vitro had increased volume densities of binucleate cells compared with placentas from embryos produced in vivo. Further studies on morphometry and histology of the bovine pathologic placenta recovered at later stages of pregnancy after somatic cloning are underway in our laboratory and will presumably give an explanation of the increased levels of PSP60 that we have observed.

Increased incidence of late hydroallantois as well as increased mean birth weight of calves derived from somatic cloning could be related to inappropriate expression of some imprinted genes [35]. In humans, it is known that overexpression of insulin-like growth factor 2 (IGF2) by loss of imprinting induces the Beckwith-Wiedeman syndrome, which is characterized by a large-size baby at birth, macroglossia, and polyhydramnios [36]. In our laboratory,

TABLE 4. Gestation period, birth weight, and postnatal survival of calves from cloned embryos.

	Group 1, somatic adult	Group 2, somatic fetal	Group 3, embryonic	Group 4, control IVF embryos
No. calves born	9	6	23	25
Gestation period (days)	277.44 ± 2.32	279.33 ± 1.08	281.26 ± 1.24	282.29 ± 1.49
Mean calving score	3.3	3.5	2.3	2.2
Mean birth weight (kg)	53.11 ± 2.01[*a]	48.83 ± 2.61[ab]	43.78 ± 1.64[b]	44.52 ± 2.08[b]
Postnatal survival >1 mo	6/9	5/6	19/23	20/25
	66.6%	83.3%	82.6%	08.0%

* Within a row, [ab] are significantly different ($P < 0.05$, F test).

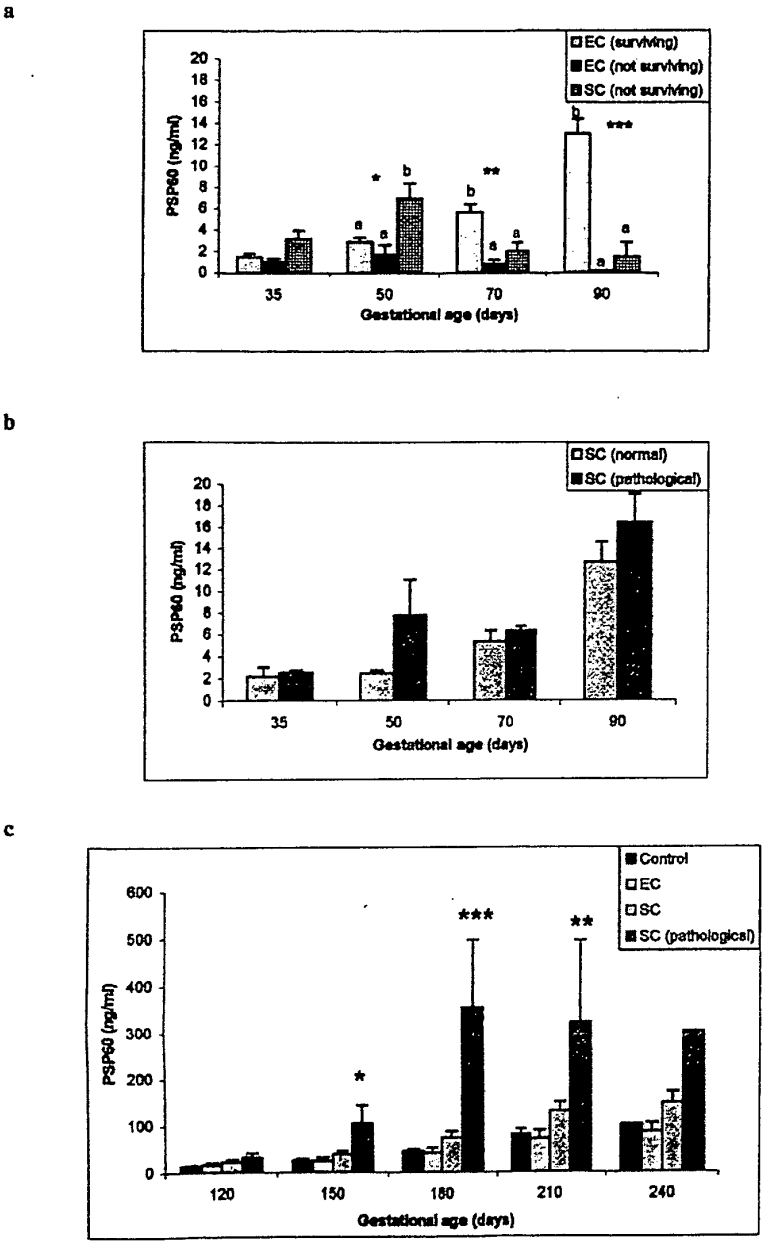

FIG. 2. PSP60 concentrations in maternal blood. a) PSP60 concentrations (mean ± SEM) in recipients carrying embryonic clones that survived, and embryonic or somatic clones that died between Days 70–90. b) PSP60 concentrations in recipients carrying somatic clones that survived beyond Day 90 according to outcome later on in pregnancy. c) PSP60 concentrations (mean ± SEM) in recipients after the first trimester of gestation. Control, Recipients pregnant after AI; EC, embryo cloning group; SC, somatic cloning group. Significant difference with other groups. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

preliminary assays suggest that the plasma concentration of IGFs and IGF binding proteins differ between neonatal clones and control calves [37]. These findings may be the consequence of abnormal growth and placental development in fetal life. In a recent study by Blondin et al. [38], it was shown that bovine fetuses originating from in vitro production systems possess altered levels of IGF mRNA in both liver and skeletal muscle. From our observations on somatic cloned fetuses recovered at slaughter after detection of hydroallantois, enlarged liver (up to 7% of body weight) was one of the characteristics of these fetuses (data not shown). Observed abnormalities may be the consequence of deregulation of imprinted genes such as IGF receptor 2, which has recently been shown to be associated with fetal overgrowth in sheep [39].

Full-term development rates after fetal or adult somatic cloning were 15% and 6.8%, respectively, and were significantly lower than in the control group after transfer of IVF embryos in the same experimental farm. These rates are comparable to those already published for somatic cloning

using fetal or adult skin fibroblasts as the source of cells [5, 40].

By the time of embryo transfer, the quality of the blastocysts as evaluated by their morphology (grades 1 and 2) was similar for the different groups, and all of them were developed in vitro under the same culture conditions [28]. Total cell counts on Day 7 blastocysts derived from embryo cloning and somatic cloning were not different from those of control IVF blastocysts. However, the proportion of ICM cells was lower in somatic cloned blastocysts [41]. This means that these four groups of in vitro-produced blastocysts of similar morphological grading can have very different potential for full-term development, although the initiated pregnancy rate as assessed by progesterone level on Day 21 is similar.

At birth, the incidence of LOS seemed to be higher for somatic clones than for embryonic clones or IVF calves. If we consider that LOS occurs when birth weight is higher than the mean birth weight plus two standard deviations, then calves weighing more than 59.5 kg at birth were considered to be large calves. The incidence of LOS at birth was 13.3% for somatic cloning, compared with 8.6% for embryonic cloning and 9.5% for the group of IVF calves. This proportion is somewhat lower than the 14.4% of calves exceeding 60 kg reported on a larger scale after in vitro embryo production [15]. After somatic cloning, the incidence of LOS could be related to the source of donor cells. According to Kato et al. [11], when donor cells were derived from cumulus and oviduct, calf body weights at birth were in the normal range, but when calves were derived from skin, ear, or liver donor cells, they observed a 47% rate of LOS (9 cases out of 19 calves born). Under our conditions, with a similar source of cells (fibroblasts derived from ear skin biopsy), our proportion of large calves was very limited (13.3%) compared with other reports. However, the maximal birth weight of our calves born in the different groups was 62 kg, and the occurrence of these large calves had no incidence on postnatal survival except for group 1, in which three calves out of nine did not survive longer than 2 mo. Postnatal survival of calves in group 1 (adult somatic clones) was lower than in all other groups, whereas the total number of animals is still limited. After embryo cloning and fetal somatic cloning, survival rates longer than 1 mo were high (>80%) and not different from those obtained for IVF calves. This is much higher than survival rates reported for embryo clones in cattle. Lewis et al. [42] reported 13 healthy calves at 2 mo from 22 full-term fetuses (60% survival), but in his experiment, twinning could have contributed to poor survival, which was not the case in the present study because only single transfers were performed. In the group of adult somatic clones, one animal died at the age of 2 mo from a thymic hypoplasia [43]. The five remaining animals are healthy and normal. Some of the females are more than 1 yr old and are cycling normally. The five animals in the group of fetal somatic clones are still alive and show no pathological manifestation. Among them, two males are already adult and have proved to be fertile. Sperm has been collected and frozen from these two bulls and is now routinely used in an IVF research program. However, according to Institut National de la Recherche Agronomique regulations, animals derived from cloning may not enter the human food chain and must be killed at the end of the experiment. This will be the case for animals in this experiment when all measurements have been made at the adult stage.

In conclusion, our experiments suggest that maternal PSP60 assays can be a good predictor of abnormal fetal development after somatic cloning in cattle, and that somatic cloning from adult cells induces higher levels of abnormalities and late losses than embryo cloning.

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REFERENCES

1. Wilmut I, Schnieke A, McWhir J, Kind AJ, Campbell KHS. Viable offspring derived from fetal and adult mammalian cells. Nature 1997; 385:810–813.
2. Kato Y, Tani T, Sotomaru Y, Kurokawa K, Kato J, Doguchi H, Yasue H, Tsunoda Y. Eight calves cloned from somatic cells of a single adult. Science 1998; 282:2095–2098.
3. Vignon X, Chesné P, LeBourhis D, Fléchon JE, Heyman Y, Renard JP. Developmental potential of bovine embryos reconstructed from enucleated matured oocytes fused with cultured somatic cells. C R Acad Sci Paris Life Sci 1998; 321:735–745.
4. Wells D, Misica P, Tervit H. Production of cloned calves following nuclear transfer with cultured adult mural granulosa cells. Biol Reprod 1999; 60:996–1005.
5. Cibelli JB, Stice SL, Golueke PJ, Kane JJ, Jerry J, Blackwell C, Ponce de Leon FAP, Robl JM. Cloned transgenic calves produced from non quiescent fetal fibroblast. Science 1998; 280:1256–1258.
6. Baguisi A, Behboodi E, Melican DT, Pollock JS, Destrempes MM, Cammuso C, Williams JL, Nims SD, Porter C, Midura P, Palacios MJ, Ayres SL, Denniston RS, Hayes ML, Ziomek CA, Meade HM, Godke RA, Gavin WG, Overström EW, Echelard Y. Production of goats by somatic cell nuclear transfer. Nature Biotechnol 1999; 17:456–461.
7. Polejaeva IA, Chen S, Vaught T, Page R, Mullins J, Ball S, Dai Y, Boone J, Walker S, Ayatres D, Colman A, Campbell K. Cloned pigs produced by nuclear transfer from adult somatic cells. Nature 2000; 407:86–90.
8. Wakayama T, Yanagimachi R. Cloning of male mice from adult tip tail cells. Nat Genet 1999; 22:127–128.
9. Zhou Q, Boulanger L, Renard JP. A simplified method for the reconstruction of fully competent mouse zygotes from adult somatic donor nuclei. Cloning 2000; 2:35–44.
10. Galli C, Duchi R, Moor R, Lazzari G. Mammalian leucocytes contain all the genetic information necessary for the development of a new individual. Cloning 1999; 1:161–170.
11. Kato Y, Tani T, Tsunoda Y. Cloning of calves from various somatic cell types of male and female adult, newborn and fetal cows. J Reprod Fertil 2000; 120:231–237.
12. Hill JR, Burghard RC, Jones K, Long C, Looney C, Shin T, Spencer T, Thompson J, Winger Q, Westhusin ME. Evidence for placental abnormality as a major cause of mortality in first-trimester somatic cell cloned bovine fetuses. Biol Reprod 2000; 63:1787–1794.
13. Heyman Y, Chavatte-Palmer P, LeBourhis D, Deniau F, Laigre P, Vignon X, Renard JP. Evolution of pregnancies after transfer of cloned bovine blastocysts derived from fetal or adult somatic cells. In: Proceedings of the 15th AETE meeting; 1999; Lyon, France: 166.
14. Hill JR, Roussel AJ, Cibelli JB, Edwards JF, Hooper NL, Miller MW, Thompson JA, Looney CR, Westhusin ME, Robl JM, Stice SL. Clinical and pathologic features of cloned transgenic calves and fetuses (13 case studies). Theriogenology 1999; 51:1451–1465.
15. Kruip TAM, den Daas JHG. In vitro produced and cloned embryos: effects on pregnancy, parturition and offspring. Theriogenology 1997; 47:43–52.
16. Walker SK, Hartwich KM, Seamark FR. The production of unusually large offspring following embryo manipulation: concept and challenges. Theriogenology 1996; 45:111–120.
17. Farin PF, Farin CE. Transfer of bovine embryos produced in vivo or in vitro: survival and fetal development. Biol Reprod 1995; 52:676–682.
18. Sinclair KD, Dunne LD, Maxfield EK, Maltin CA, Young LE, Wilmut I, Robinson JJ, Broadbent PJ. Fetal growth and development following temporary exposure of Day 3 ovine embryos to an advanced uterine environment. Reprod Fertil Dev 1998; 10:263–269.

19. Heyman Y, Marchal J, Chesne P, LeBourhis D, Deniau F, Renard JP. Outcome of pregnancy after single or twin transfer of cloned embryos in cattle. In Proceedings of the 13th AETE meeting; 1997; Lyon, France: 156.
20. Prior RL, Laster DB. Development of the bovine fetus. J Anim Sci 1979; 48:1546–1553.
21. Hubbert WT, Stalheim OVH, Booth GD. Changes in organ weights and fluid volumes during growth of the bovine fetus. Growth 1972; 36:217–233.
22. Renaudin CD, Gillis CL, Tarantal AF, Coleman DA. Ultrasonographic evaluation of equine fetal growth from 100 days of gestation to parturition. In: Proceedings of the VIIth International Symposium on Equine Reproduction; 1998; Pretoria, South Africa: 171–172.
23. Sasser RG, Ruder CA, Ivani KA, Butler JE, Hamilton WC. Detection of pregnancy by radioimmunoassay of a novel pregnancy-specific protein in serum of cows and a profile of serum concentrations during gestation. Biol Reprod 1986; 35:936–942.
24. Zoli AP, Guilbault LA, Delahaut P, Benitez Ortiz W, Beckers JF. Radioimmunoassay of a bovine pregnancy associated glycoprotein in serum: its application for pregnancy diagnosis. Biol Reprod 1992; 46: 83–92.
25. Mialon MM, Camous S, Renand G, Martal J, Menissier F. Peripheral concentration of a 60-kDa pregnancy specific protein during gestation and after calving in relationship to embryonic mortality in cattle. Reprod Nutr Dev 1993; 33:269–282.
26. Chesné P, Heyman Y, Peynot N, Renard JP. Nuclear transfer in cattle: birth of cloned cattle and estimation of blastomere totipotency in morulae used as source of nuclei. C R Acad Sci 1993; 316:487–491.
27. Heyman Y, Chesné P, Le Bourhis D, Peynot N, Renard JP. Developmental ability of bovine embryos after nuclear transfer based on the nuclear source: in vivo versus in vitro. Theriogenology 1994; 42:695–702.
28. Menck C, Guyader-Joly C, Peynot N, Le Bourhis D, Lobo RB, Renard JP, Heyman Y. Beneficial effect of Vero cells for developing IVF bovine eggs in two different coculture systems. Reprod Nutr Dev 1997; 37:141–150.
29. Revel F, Mermillod P, Peynot N, Renard JP, Heyman Y. Low development capacity of in vitro matured and fertilized oocytes from calves compared with that of the cows. J Reprod Fertil 1995; 103:115–120.
30. Pipers FS, Adams-Brendemuehl CS. Techniques and applications of transabdominal ultrasonography in the mare. J Am Vet Med Assoc 1994; 185:766–771.
31. Reef VB, Vaala WE, Worth LT, Sertich PL. Ultrasonographic assessment of fetal well-being during late gestation: development of an equine biophysical profile. Equine Vet J 1996; 28:200–208.
32. Young LE, Fairburn HR. Improving the safety of embryo technologies: possible role of genomic imprinting. Theriogenology, 2000; 53: 627–648.
33. Vasques ML, Horta AEM, Marques CC, Sasser RG, Humblot P. Levels of bPSPB throughout single and twin pregnancies after AI or transfer of IVM/IVF cattle embryos. Anim Reprod Sci 1995; 38:279–289.
34. Farin PW, Stewart RE, Rodriguez KF, Crozier AE, Blondin P, Alexander JE, Farin CE. Morphometry of the bovine placenta at 63 days following transfer of embryos produced in vivo or in vitro [abstract]. Theriogenology 2001; 55:320.
35. Kono T. Influence of epigenetic changes during oocyte growth on nuclear reprogramming after nuclear transfer. Reprod Fertil Dev 1998; 10:593–598.
36. Reik W, Brown KW, Schneid H, Le Bouc Y, Bickmore W, Maher ER. Imprinting mutations in the Beckwith-Wiedeman syndrome suggested by altered imprinting pattern in the IGF2-H19 domain. Hum Mol Genet 1995; 4:2379–2385.
37. Chavatte-Palmer P, Heyman Y, Monget P, Richard C, Kann G, LeBourhis D, Vignon X, Renard JP. Plasma IGF-1, IGFBP, and GH concentrations in cloned neonatal calves from somatic and embryonic cells: preliminary results [abstract]. Theriogenology 2000; 53:213.
38. Blondin P, Farin PW, Crosier AE, Alexander JE, Farin CE. In vitro production of embryos alters levels of insulin-like growth factor-II messenger ribonucleic acid in bovine fetuses 63 days after transfer. Biol Reprod 2000; 62:384–389.
39. Young LE, Fernandes K, McEvoy TG, Buttewith SC, Gutierrez GC, Carolan C, Broadbent PJ, Robinson JJ, Wilmut I, Sinclair DS. Epigenetic changes in IGFR2 is associated with fetal overgrowth after sheep embryo culture. Nat Genet 2001; 27:153–154.
40. Kubota C, Yamakuchi H, Todoraki J, Mizoshita K, Tabara N, Barber M, Yang X. Six cloned calves produced from adult fibroblast cells after long term culture. Proc Natl Acad Sci U S A 2000; 97:990–995.
41. LeBourhis D, Lavergne Y, Vignon X, Heyman Y, Renard JP. Comparison of bovine blastocyst quality after somatic nuclear transfer or in vitro fertilisation. In: Proceedings of the 16th AETE meeting, Santander; 178. 2000
42. Lewis IM, Peura TT, Owens JL, Ryan MF, Diamente MG, Pushett DA, Lane MW, Jenjkin G, Coleman PJ, Trounson AO. Outcomes from novel simplified nuclear transfer techniques in cattle. Theriogenology 2000; 53:233.
43. Renard JP, Chastant S, Chesne P, Richard C, Marchal J, Cordonnier N, Chavatte P, Vignon X. Lymphoid hypoplasia and somatic cloning. Lancet 1999; 353:1489–1491.

附件二.

Early report

Lymphoid hypoplasia and somatic cloning

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Summary

Background Adult somatic cloning by nuclear transfer is associated with high rate of perinatal mortality but there is still no evidence that nuclear transfer itself is responsible for these failures. We report on a longlasting defect linked to somatic cloning.

Methods Skin cells grown from an ear biopsy specimen from a 15-day-old calf were used as a source of nuclei. The donor animal was a clone of three females obtained from embryonic cells. Clinical examination, haematological, and biochemical profiles, and echocardiography of the somatic clone were done from birth to death.

Findings After 6 weeks of normal development, the somatic cloned calf had a sudden and rapid fall in lymphocyte count and a decrease in haemoglobin. The calf died on day 51 from severe anaemia. Necropsy revealed no abnormality except thymic atrophy and lymphoid hypoplasia.

Interpretation Somatic cloning may be the cause of long-lasting deleterious effects. Our observation should be taken into account in debates on reproductive cloning in human beings.

Lancet 1999; **353:** 1489–91

Introduction

Cloning of mammals involves the introduction of a nucleus into an enucleated recipient oocyte. In mammals, cloning produces live and normal offspring with embryonic cells as a source of nuclei.[1,2] Nuclear transfer was then successfully extended to cultured embryonic donor cells,[3-5] then to somatic nuclei from cultured foetal cells.[6-8] Cloning of mammals from adult somatic cells was then achieved in sheep,[6] and recently extended to mice,[9] and to cattle.[10]

Cloning has a relatively high rate of late abortion and early postnatal death, particularly when somatic cells are used as donors of nuclei; rates as high as 40–74% have been reported.[5,7,8,10] It is not known if these developmental failures are due to nuclear reprogramming or to the cloning procedure itself. Culture has been shown to affect the viabilty of offspring through unclear epigenetic mechanisms.[11] For instance, culture of preimplantation sheep and cattle embryos is frequently associated with an abnormally high birth weight ("large offspring syndrome").[12-13]

We report that adult somatic cloning may be responsible for developmental failures occurring after birth.

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Methods

Donor cells were obtained from a surface biopsy specimen taken from the ear of a 15-day-old calf. This donor animal was a nuclear transfer clone obtained by the fusion of an embryonic cell (blastomere) with an enucleated oocyte. The skin biopsy specimen was allowed to grow in vitro for 10 days in DMEM supplemented with 10% foetal calf serum before being subcultured; the resulting cell cultures were frozen after three passages. For nuclear transfer, the cells were thawed and those from batches karyotyped positively (60 chromosomes; 85% of cells) were used between passage 6 to 10 (about 20 to 30 days) of in-vitro culture.

Receipient oocytes were prepared by enucleating after 24 h of in-vitro maturation followed by an ageing period of 10 h at 10°C in TCM-199 before embryo reconstitution.[14]

Cloned embryos were obtained by fusing a donor cell with an enucleated oocyte through an electrostimulation of two pulses of 1·5 kV/cm for 50 µs in a 0·3 mol/L mannitol solution containing 0·1 Ca^{++} and Mg^{++}. Fused embryos were transferred 1 h later in microdrops of B2 medium with 10% FCS and co-cultured with Vero cells under mineral oil. On day 7 of in-vitro culture, the embryos that had developed to an apparently normal blastocyst were used for non-surgical transfer into recipient heifers. Pregnancy was diagnosed by ultrasonographic examination on day 35. The cow was induced with dexamethasone at term (276 days of pregnancy) and the calf delivered by caesarian section. The calf was given thawed colostrum as a routine. Genomic DNA from the donor cells, the newborn calf and the recipient heifer were typed for miscrosatellites to ascertain the origin of the clone. Blood samples were taken from the calf every 2 days after birth for haematological monitoring.

Results

The first calf was born on July 6, 1998. It had been cloned from a nucleus obtained from thawed frozen cells grown in vitro for 4 weeks (8 passages). 175 nuclear transfer

Figure 1: **Cloned calf at 6 weeks and its cell donor at 14 months of age**

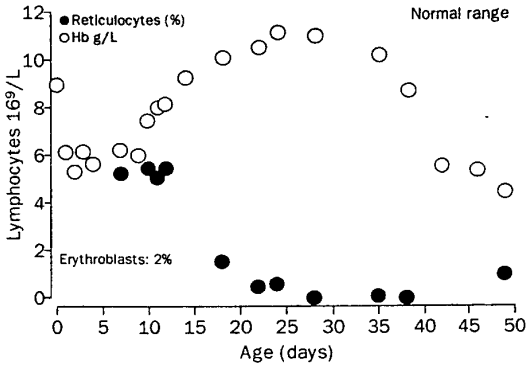

Figure 2: **Haemoglobin concentration, reticulocytes, and erythroblast counts**

embryos were reconstructed from which six blastocysts were transplanted in five recipient females. One recipient was diagnosed as pregnant by ultrasound examination on day 35, and gave birth to a calf whose genotype was confirmed by microsatellite analysis as being the same as the donor.

Echocardiography was done immediately after birth and revealed an enlarged right ventricle. Treatment with an ACE inhibitor and diuretics was given for 1 week, and the condition resolved. No further cardiac defects were detected by ultrasound. There were no further problems during the first month after birth during which the calf developed normally (figure 1).

Haematological monitoring showed a high reticulocyte count (up to 5%), and erythroblasts in the blood within the first 3 weeks (figure 2). Lymphocyte counts were normal until about 1 month after birth; they then rapidly fell (figure 3). Haemoglobin concentration also decreased abruptly after 40 days without a concomitant increase in reticulocytes. Iron supplements had no effect on haemoglobin.

The calf died on day 51 from severe anaemia. No evidence of infection, malformation, or phenotypical abnormality was found at necropsy, except marked thymic atrophy. Histological examination revealed lymphoid aplasia. The thymus (figure 4), spleen, and lymph nodes were hypoplastic. The lack of secondary follicles in the lymph nodes together with the reduced number of primary follicles, the reduced size of their paracortex, and the presence of collapsed medullary cords containing few plasma cells suggested that hypoplasia was present at birth. The presence of Bovine Viral Diarrhoea virus, known to induce thymic atrophy, was excluded by PCR of donor cells and of calf serum. Analysis of IgG from stored aliquots of sampled blood showed marked increases of IgG

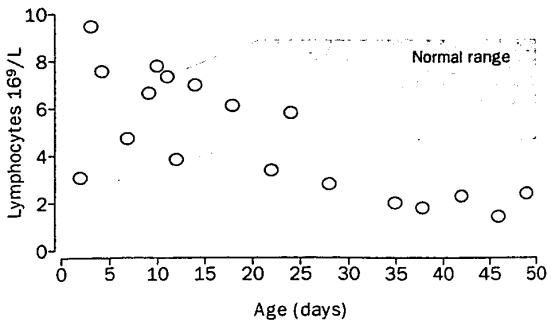

Figure 3: **Lymphocyte counts**

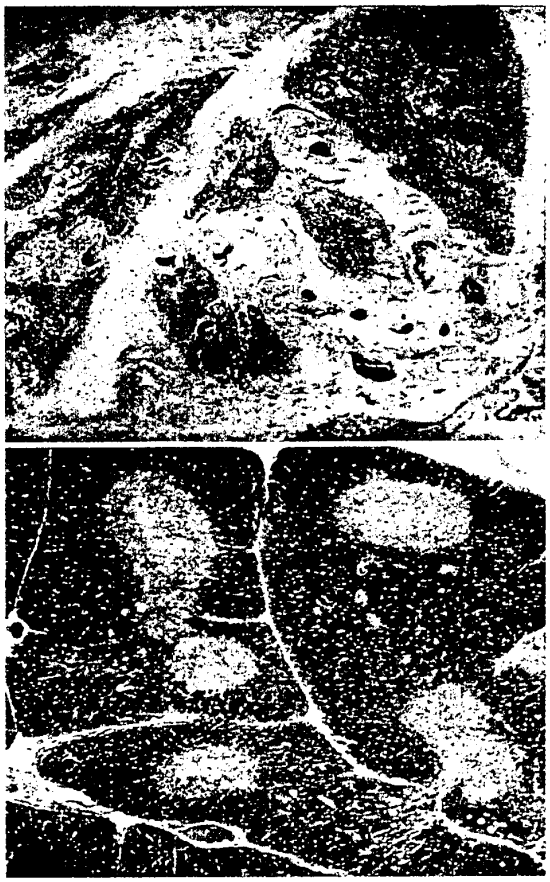

Figure 4: **Histological section of thymus with noticeable lymphoic depletion (A) compared with a normal thymus (B)**
Final magnification ×85.

at birth due to the absorption of colostrum but a rapid subsequent decrease to constantly low concentrations (lower than 5 g/L, data not shown) without any clear evidence of endogeneous synthesis.

Discussion

We describe an animal born by adult somatic cloning which started to grow normally but then died at the age of 7 weeks. Unlike previous reports,[15] no circulatory abnormality was found at necropsy. Haematological monitoring suggested that anaemia was the cause of death and histology showed global lymphoic hypoplasia. This is the first report of a long-lasting defect associated with somatic cloning. Since the birth of the first somatically cloned sheep, two reports have been published on development after nuclear transfer of adult somatic nuclei.[9,10] In those reports a high proportion of animals died during late gestation or within the first days of birth. Prenatal and perinatal losses have also been reported with cloning using foetal cells.[6,7] It is not known whether these failures were due to somatic cloning or to other factors which include in-vitro culture conditions of recipient oocytes and of the reconstructed embryos, the nuclear transfer technique, and possible existence of some lethal defective genotype in the donor animal.

Since in our study the donor animal was itself generated by embryonic cloning, these other factors can be ruled out.

The same culture conditions and nuclear-transfer technique were used to produced the cell-donor animal and its somatic clone. The same protocol has produced in our laboratory more than a hundred healthy calves from embryonic donor cells.[16] Lethal genotype can also be excluded because the cell-donor animal belongs to a group of three embryonic clones that are currently healthy and are now older than 1½ years. Factors inherent to somatic cloning—i.e, the random choice of a cultured donor cell that bears a mutation—or the lack of fine-tuned regulation during somatic reprogramming can be considered as the cause of death.

Thymic hypoplasia has already been observed in one of our previous clones obtained from a fetal muscle cell.[8] The clone died at about the same age from an acute and general infection after accidental trauma. We could not determine if the observed deficiency was a consequence of or preceded the accident, but we were very surprised by the rapid extension of the infectious process despite aggressive and immediate antibiotics. Similar cloning conditions have produced normal calves both from skin and muscle fetal cells. These calves are now between 2 and 12 months old.

A report on eight calves born from ten embryos with somatic nuclei of a single adult cow reinforces the interest of cloning for animal selection and transgenesis.[10] This increase in cloning efficiency is associated with a high mortality rate of offspring (50%). Long-lasting effects triggered by nucleocytoplasmic interactions at the one-cell stage have been shown in mice.[17] Our report suggests that such epigenetic events can also affect the correct reprogramming of gene activity. This, together with the fact that uncontrolled defective gene regulation induced by nuclear transfer can, in the mouse, be transmitted to offspring,[18] should be taken into account in debates on the effective application of reproductive somatic cloning to human beings.

Contributors

Jean-Paul Renard was the advisor for the study and contributed to writing of the paper. Patrick Chesné did cloning work.
Christophe Richard and Jacques Marchal were responsible for animal handling, and Nathalie Cordonnier did histological preparations and observations. Xavier Vignon was in charge of preparation of donor cells, Sylvie Chastant undertook the necropsy and Pascale Chavatte was responsible for clinical examinations and blood monitoring. All authors helped in the preparation of the paper.

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References

1 Willadsen SM. Nuclear transplantation in sheep embryos. *Nature* 1986; **320:** 63–65.
2 Prather RS, Barnes FL, Sims MN, Robl JM, Eyestone WH, First NL. Nuclear transplantation in the bovine embryo: assessment of donor nuclei and recipient oocyte. *Biol Reprod* 1987; **37:** 859–6.
3 Sims M, First NL. Production of calves by transfer of nuclei from cultured inner cell mass cells. *Proc Natl Acad Sci USA* 1993; **90:** 6143–47.
4 Campbell KHS, McWhir J, Ritchie WA, Wilmut I. Sheep cloned by nuclear transfer from a cultured cell line. *Nature* 1996; **380:** 64–6.
5 Wells DN, Misica PM, Day AM, Tervit HR. Production of cloned lambs from an established embryonic cell line: a comparison between in-vivo- and in-vitro-matured cytoplasts. *Biol Reprod* 1997; **57:** 385–93.
6 Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KHS. Viable offspring derived from fetal and adult mammalian cells. *Nature* 1997; **385:** 810–13.
7 Cibelli JB, Stice SL, Golueke PJ, et al. Cloned transgenic calves produced from non-quiescent fetal fibroblasts. *Science* 1998; **280:** 1256–58.
8 Vignon X, Chesné P, Le Bourhis D, Flechon JF, Heyman Y, Renard JP. Developmental potential of bovine embryos reconstructed from enucleated matured oocytes fused with cultured somatic cells. *C R Acad Sci* 1998; 321: 735–45.
9 Wakayama T, Perry ACF, Zuccotti M, Johnson KR, Yanagimachi R. Full term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature* 1998; **394:** 369–74.
10 Kato Y, Tani T, Sotomaru Y, et al. Eight calves cloned from somatic cells of a single adult. *Science* 1998; 282: 2095–098.
11 Young LE, Sinclair KD, Wilmut I. Large offspring syndrome in cattle and sheep. *Reviews in Reproduction* 1998; **3:** 155–63.
12 Gary FB, Adams R, Mccann JP, Odde KG. Postnatal characteristics of calves produced by nuclear transfer cloning. *Theriogenology* 1996; **45:** 141–52.
13 Kruip TAM, Den Daas JHG. In vitro produced and cloned embryos: effects on pregnancy, parturition and offspring. *Theriogenology* 1997; **47:** 43–52.
14 Chesné P, Heyman Y, Peynot N, Renard JP. Nuclear transfer in cattle: birth of cloned cattle and estimation of blastomere totipotency in morulae used as source of nuclei. *C R Acad Sci* 1993; **316:** 487–91.
15 Hill JR, Roussel AJ, Edwards JF, Thompson JA, Cibelli JB. Clinical and pathologic features of cloned transgenic calves and fetuses (13 cases: 1997–1998). Proceedings of the annual meeting of the Society for Theriogenology December 4–6, 1998 Baltimore, Maryland, USA.
16 Heyman Y, Renard JP. Cloning of domestic species. *Anim Reprod Sci* 1996; **42:** 427–36.
17 Reik W, Römer I, Barton SC, Surani MA, Howlett SK, Klose J. Adult phenotype in the mouse can be affected by epigenetic events in the early embryo. *Development* 1993; **119:** 933–42.
18 Römer I, Reik W, Dean W, Klose J. Epigenetic inheritance in the mouse. *Curr Biol* 1997; **77:** 277–80.

Case report

Sunshine, sweating, and *main d'accoucheur*

David W Johnson, Alan Parnham, Karen Herzig, Johannes Wittmann

A 33-year-old man was admitted to hospital in March, 1998, with tetany and faintness. He had been seen twice over the previous 5 years with the same symptoms and on both occasions had been found to have unexplained hypokalaemia (serum potassium below 2 mmol/L) with a metabolic alkalosis. He had no history of vomiting, diarrhoea, use of diuretics, hyperventilation, or symptoms of sinus or lung disease. His tetany was precipitated by manual labour under sweltering conditions. Since early childhood, he had sweated a lot and often left a salt precipitate on his clothing. A family history was not available because he was adopted. His only child had been conceived by artificial insemination of donor semen because of obstructive azoospermia due to congenital bilateral absence of the vas deferens. Physical examination showed a blood pressure of 70/40 mm Hg, dehydration, sweating, and bilateral *main d'accoucheur*. Cardiorespiratory and abdominal examinations were normal. His body-mass index was 26 kg/m^2. His plasma biochemistry is shown in the table. A urine sample had a potassium concentration of 76·4 mmol/L and a chloride concentration of less than 20 mmol/L. An electrocardiogram was normal apart from a prolonged QTc interval (0·52 s). After rehydration with intravenous saline and potassium supplements, his symptoms were relieved; and electrolyte abnormalities, renal functional impairment, and electrocardiographic abnormalities returned to normal. Subsequent investigations of thyroid function, plasma renin activity, plasma aldosterone concentration, and 24 h urinary free cortisol were normal.

Measurement of sweat electrolytes by pilocarpine iontophoresis showed chloride, sodium, and potassium concentrations of 179, 190, and 24 mmol/L (normal values: <60, <70, <20). Screening for ten of the common mutations of the cystic fibrosis transmembrane conductance regulator gene *(CFTR)* (ΔF508, G551D, R553X, G542X, N1303K, A455E, 621+1, ΔI507, and R117H), showed a single ΔF508 mutation. Pulmonary function tests showed mild to moderate, bronchodilator-unresponsive airflow limitation with some gas trapping: forced expiratory volume in 1 s (FEV_1) 3·33 L (80% predicted), forced vital capacity (FVC) 5·11 L (102% predicted), FEV_1/FVC 65%, middle forced expiratory flow rate 2·14 L/s (45%). A chest radiograph showed clear lung fields. There was no evidence of pancreatic disease. He was treated with sodium chloride tablets and remained well until 9 months later when he had a fourth episode of saline-responsive syncope and hypokalaemic metabolic alkalosis on a hot summer's day.

Electrolyte	Concentration (mmol/L)
Sodium	130
Potassium	1·7
Chloride	78
Bicarbonate	35
Calcium	2·55
Magnesium	0·8
Urea	10·2
Creatinine	0·16

Patient's plasma biochemistry on admission to hospital

The combination of exertional saline-responsive metabolic alkalosis, low urinary chloride concentration, abnormal sweat test, aplasia of the vas deferens, and a detectable ΔF508 *CFTR* mutation indicate that this patient's electrolyte derangements occurred as a result of excessive sweat chloride losses due to an atypical form of cystic fibrosis. Hypokalaemic, hypochloraemic metabolic alkalosis is a recognised complication of cystic fibrosis[1,2] and has been attributed to impaired transport of chloride via a cAMP-regulated channel (the *CFTR*) leading to excessive sweat chloride and potassium losses, volume depletion, secondary hyperaldosteronism, and alkalosis-induced redistribution of potassium from extracellular to intracellular fluid.[1-3] Since the discovery of the *CFTR* gene in 1989, over 750 mutations have been identified. The most common of these, ΔF508, accounts for 70% of known cystic fibrosis chromosomes, while commercially available screening panels detect up to 85% of the known alleles.[3] The ability to screen for *CFTR* mutations has further led to the recognition that about 2% of adults present with an atypical form of disease, including nasal polyposis, pancreatitis, and congenital absence of the vas deferens.[3] In adult men with vas deferens aplasia, the frequency of ΔF508 heterozygosity is more than six times the expected population frequency of cystic fibrosis gene carriers.[4] Despite normal pulmonary and gastrointestinal function, up to 25% of these patients also have abnormal sweat tests.[4,5] It has been postulated that these patients represent compound heterozygotes with a different, as yet unidentified, mutation on the other allele leading to a form of cystic fibrosis the effects of which are limited to genital defects and abnormal sweating. This report suggests that recurrent, unexplained hypokalaemic metabolic alkalosis, particularly with other clinical manifestations of *CFTR* mutations (such as obstructive azoospermia), should prompt consideration of the diagnosis of cystic fibrosis.

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References

1 Kennedy JD, Dinwiddie R, Daman-Willems C, Dillon MJ, Matthew DJ. Pseudo-Bartter's syndrome in cystic fibrosis. *Arch Dis Child* 1990; 65: 786–87.

2 Devlin J, Beckett NS, David TJ. Elevated sweat potassium, hyperaldosteronism and pseudo-Bartter's syndrome: a spectrum of disorders associated with cystic fibrosis. *J Roy Soc Med* 1989; 82 (suppl 16): 38–43.

3 Durie PR. Pancreatitis and mutations of the cystic fibrosis gene. *N Engl J Med* 1998; 339: 687–88.

4 Rigot JM, Lafitte JJ, Dumur V, et al. Cystic fibrosis and congenital absence of the vas deferens. *N Engl J Med* 1991; 325: 64–65.

5 Anguiano A, Oates RD, Amos JA, et al. Congenital bilateral absence of the vas deferens: a primarily genital form of cystic fibrosis. *JAMA* 1992; 267: 1794–97.

附件三

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Piglets Born after Vitrification of Embryos Using the Open Pulled Straw Method[1]

Françoise Berthelot,[2] Françoise Martinat-Botté, A. Locatelli, Christine Perreau, and M. Terqui

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Morulae and unhatched blastocysts from Large White hyperprolific (LWh) and Meishan (MS) gilts were selected to test an ultrarapid open pulled straw (OPS) vitrification method with two media. The viability of vitrified/warmed embryos was estimated by the percentage of embryos that developed to the hatched blastocyst stage *in vitro* or by birth after transfer. In Experiment 1, two cryoprotectant dilution media were compared for cryopreservation of MS and LWh blastocysts: TCM was a standard Hepes-buffered TCM199 + 20% NBCS medium and PBS was a PBS + 20% NBCS medium. After a two-step equilibration in ethylene glycol, dimethyl sulfoxide, and sucrose, 2–5 blastocysts were loaded into OPS and plunged into liquid nitrogen. Embryos were warmed; a four-step dilution with decreasing concentrations of sucrose was applied. In PBS, LWh blastocysts (27%) had a lower viability *in vitro* than MS blastocysts (67%; $P = 0.001$). In TCM, no significant difference was observed between genotypes (41% for LWh and 43% for MS blastocysts) and both viability rates were lower than that of the control groups. In Experiment 2, morula-stage LWh and MS embryos were vitrified and warmed using PBS. The viability rate was low and did not differ between LWh (11%) and MS (14%). In Experiment 3, 200 MS and 200 LWh blastocysts were vitrified/warmed as described in Experiment 1 (PBS). In each of 20 MS recipients, 20 embryos were transferred. The farrowing rate was 55% and recipients farrowed four and five piglets (median) for MS and LWh blastocysts, respectively. The OPS method is therefore appropriate for cryopreservation of unhatched porcine blastocysts. © 2000 Academic Press

Key Words: pig; vitrification; OPS; morula; blastocyst; genotype; *in vitro* development; embryo transfer.

Porcine embryos are known to be more sensitive to damage caused by cryopreservation than other mammal embryos (7, 26). Only a few piglets have been born after transfer of slow-rate frozen and thawed embryos (21, 22). The presence of a large number of intracytoplasmic lipid droplets has often been mentioned as an obstacle to successful freezing of porcine embryos. Removal of these lipid droplets may increase survival rates: three piglets were born after the transfer of 181 delipated frozen/thawed embryos (23). However, none of the methods proposed have given satisfactory results. Moreover, they are often difficult to implement. In addition, most of the results achieved have not been confirmed in later experiments. Conventional freezing therefore does not seem to be suitable for preservation of porcine embryos.

Vitrification is currently being studied in several species to resolve the problems of cryopreservation. To totally eliminate ice crystal formation, embryos are equilibrated with high concentrations of cryoprotectants and then plunged immediately into liquid nitrogen. Using traditional 250-μl insemination straws, the fastest achievable cooling rate is approximately 2500°C/min, which allows embryos to pass through certain critical temperature zones quickly and decreases chilling injuries (27). With pigs, this method has so far resulted in the birth of 10 piglets after transfer of 157 cytoskeletal-stabilized and vitrified/thawed hatched blastocysts. Twenty-nine piglets were also born after the transfer of 224 cytoskeletal-stabilized hatched blastocysts (8, 9). Another team obtained 11 piglets after 17 transfers of 16 to 30 expanded and hatched blastocysts (12, 13). However, only 1 successful transfer (4 piglets)

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0011-2240/00 $35.00

→ 35% of the embryo
70–85% PR after 20 ET.

of exclusively unhatched blastocysts has so far been reported (13).

Several new techniques have recently been developed to increase the cooling and warming rates of vitrification, including the use of electron microscopic grids to provide support to oocytes and embryos during manipulations (19, 31) and open pulled straw (OPS) technology. The low volume of cryoprotectant medium surrounding oocytes or embryos and the direct contact with liquid nitrogen provide very high cooling rates (approx 24,000°C/min with OPS technology). These methods have been successfully used for vitrification of oocytes and embryos in cattle, and high *in vitro* survival rates were achieved after cryopreservation of embryos from several other species, including pigs (11, 15, 19, 31, 33, 35). The faster cooling rate seems to be one of the key solutions to protecting porcine embryos from chilling injuries and obtaining piglet births after vitrification of unhatched blastocysts (1, 2). Our preliminary results (2) confirm this observation.

In this study, we attempted to confirm the efficiency of rapid cooling performed with OPS vitrification (33, 35). Two cryoprotectant dilution media were compared to determine whether saline concentration or osmotic pressure had an effect on the blastocyst survival. As differences in embryonic development have been observed in Meishan and Large White or crossbred embryos (10, 32), we tested these two cryoprotectant media on Meishan (MS) and Large White hyperprolific (LWh) embryos to ensure that the method used was not specific to a given genotype. To complete the study, the most efficient cryoprotectant media were used for vitrification of morula-stage Meishan and Large White hyperprolific embryos. Finally, one of the methods was selected to evaluate *in vivo* blastocyst development of these two genotypes after vitrification, warming, and transfer to recipients.

MATERIALS AND METHODS

Animals

Embryo donors and recipients came from the INRA experimental pig herd in Nouzilly, France. They were cyclic gilts aged 5 to 8 months at the time of their introduction into the experiments. Donors were Meishan genotype ($n = 62$) or Large White hyperprolific genotype ($n = 63$) (5). Recipients were Meishan ($n = 20$).

Embryo Production and Collection

Embryo production. Estrus detection was checked twice per day with a boar. Embryo donors underwent double insemination at an interval of 12–24 h at a spontaneous estrus. The semen collections and doses were prepared by the INRA Experimental Artificial Insemination Center (Station Expérimentale d'Insémination Artificielle, Rouillé, France). Donor females were inseminated twice with 3×10^9 spermatozoa per insemination, using semen from a boar of a different genotype (mostly Piétrain) to benefit from heterosis (16). The maternal genotype of the embryo is indicated only when discussing embryo genotypes.

Embryo collection. Donor gilts were slaughtered 5 to 6 days after the first insemination, and their reproductive tracts were immediately removed. Embryos were collected after flushing the uterine horns with phosphate buffer at 39°C containing 2% newborn calf serum (NBCS) from GIBCO-BRL, France. Embryo development stages were evaluated under a stereomicroscope, with 20× magnification. Only morulae and/or unhatched blastocysts were selected.

Embryo culture and survival evaluation. In vitro development of control and vitrified/warmed embryos was carried out at 39°C in an atmosphere containing 5% CO_2, 5% O_2, and 90% N_2 in 100 μl of culture medium, without polyvinyl pyrrolidone but with 20% fetal calf serum (FCS) added (GIBCO–BRL) (3). This medium was adapted from UB medium, derived from NCSU23 medium (24). FCS was always used instead of NBCS for *in vitro* culture of embryos. The duration of culture was 2 to 5 days, depending on the initial stage of the embryo.

Only embryos that hatched after a maximum of 5 days were considered surviving. The hatch-

TABLE 1
Experimental Outline

Exp.	Genotype	Stage	T (°C)	Medium (Med)	Vitrification	Time (min)	Warming	Time (min)	Development
1	LWh MS	Unhatched blastocyst	39	TCM or PBSx1 + PBSx2	Med alone	1	Med + 0.2 M Suc	1	*In vitro*
					Med alone	1	Med + 0.2 M Suc	5	
					Med + 1 M Me_2SO + 1.3 M EG	3	Med + 0.1 M Suc	5	
							Med alone	5	
					Med + 2.5 M Me_2SO + 3.2 M EG + 0.6 M Suc	1			
2	LWh MS	Morula	39	PBSx1 + PBSx2	Med alone	1	Med + 0.2 M Suc	1	*In vitro*
					Med alone	1	Med + 0.2 M Suc	5	
					Med + 1.4 M Me_2SO + 1.8 M EG	3	Med + 0.1 M Suc	5	
							Med alone	5	
					Med + 2.8 M Me_2SO + 3.6 M EG + 0.6 M Suc	1			
3	LWh MS	Unhatched blastocyst	39	PBSx1 + PBSx2	Med alone	1	Med + 0.2 M Suc	1	*In vivo*
					Med alone	1	Med + 0.2 M Suc	5	
					Med + 1 M Me_2SO + 1.3 M EG	3	Med + 0.1 M Suc	5	
							Med alone	5	
					Med + 2.5 M Me_2SO + 3.2 M EG + 0.6 M Suc	1			

Note. LWh, Large White hyperprolific; MS, Meishan; Suc, sucrose; TCM, Hepes TCM199 + 20% NBCS; PBSx1, PBS + 20% NBCS; PBSx2, PBS double concentration + 20% NBCS.

ing rate is shown as a percentage of the total number of embryos vitrified or observed.

Experimental Protocol

An experimental outline for the three experiments is shown in Table 1.

Experiment 1: Effect of Vitrification Medium and Genotype of Blastocysts on Hatching Rate after in Vitro Development

Two cryoprotectant dilution media were tested to determine their effects on blastocyst survival for the two genotypes:

(a) PBS, which was a Dulbecco's phosphate-buffered saline medium adjusted to 290 mOsm using double concentration (PBS × 2) to compensate for the saline dilution due to the high concentration of cryoprotectants.

(b) TCM, which was a Hepes-buffered TCM199 medium.

Both media were supplemented with 20% NBCS. The cryoprotectants used were dimethyl sulfoxide (Me_2SO) and ethylene glycol (EG) (35). These two media and the cryoprotectants were purchased from Sigma (France) and OPS was purchased from Szigta Co., (Clayton, Australia).

At vitrification, Large White hyperprolific and Meishan blastocysts were randomly allocated among the two media. Vitrification was performed using OPS in groups of two to five blastocysts. All equilibrations were performed at 39°C.

Blastocysts were equilibrated successively in two subsequent baths of TCM or PBS for 1 min and then in

- Cryoprotectant 1: 1 M Me_2SO + 1.3 M EG diluted in TCM or PBS for 3 min.
- Cryoprotectant 2: 2.5 M Me_2SO + 3.2 M EG + O.6 M sucrose diluted in TCM or PBS for 1 min.

Blastocysts were then drawn up together in a 2-μl drop of Cryoprotectant 2 medium measured with a pipette, gently placed in the bottom of a culture dish, and loaded into OPS with the narrow end. The OPS used was a French ministraw, heat-softened, pulled, and cut at the narrowest point to reduce the diameter at one end

to approximately half of the original. Due to the capillary effect, the 2-μl drop with the embryos was immediately loaded into the straw and then submerged into liquid nitrogen (33).

After removal from the liquid nitrogen, straws were maintained in ambient air for 5 s before the narrow end was immersed in the first rehydration solution at 39°C. The embryos were gradually removed from the straw by gravity. The cryoprotectant dilution was made by passing the embryos through three successive dilution solutions:

- Rehydration 1: TCM or PBS + 0.2 M sucrose for 1 min.
- Rehydration 2: TCM or PBS + 0.2 M sucrose for 5 min.
- Rehydration 3: TCM or PBS + 0.1 M sucrose for 5 min and then for 5 min in TCM or PBS.

All blastocysts were then transferred to culture medium and cultured as described earlier to evaluate their capacity to hatch.

Experiment 2: Effect of Developmental Stage and Genotype

Morula-stage Large White hyperprolific and Meishan embryos were vitrified in this experiment, using PBS. The embryos were vitrified and warmed using the same procedure as that described for Experiment 1, but cryoprotectant concentrations were different from those in Experiment 1 and corresponded to those indicated by G. Vajta (personal communication).

Embryos were equilibrated at 39°C successively in the following media: twice in PBS for 1 min and then in

- Cryoprotectant 1: PBS + 1.4 M Me_2SO + 1.8 M EG for 3 min.
- Cryoprotectant 2: PBS + 2.8 M Me_2SO + 3.6 M EG + 0.6 M sucrose for 1 min.

Warming and embryo culture were performed as described for Experiment 1.

Experiment 3: Viability of Vitrified/Warmed Meishan or Large White Hyperprolific Blastocysts after Transfer

Large White hyperprolific (n = 200) and Meishan (n = 200) blastocysts, vitrified/warmed as described for Experiment 1 and without selection, were transferred to 20 Meishan recipients, 1 to 2 h after warming and subsequent *in vitro* culture (20 blastocysts to each recipient). Blastocysts were collected in 50 μl culture medium in a Teflon embryo catheter (diameter 1 mm) connected to a 1-ml syringe and then transferred into a recipient. Recipients were Meishan gilts, since it has been shown that gestation and embryo survival rates are higher with this genotype (20).

No recipients were inseminated. The transfer was performed surgically (16) and asynchronously (−24 h) with gilts relative to donors. This means that the onset of estrus of the recipients appeared 24 h after that of the donors. Embryos were transferred after a midventral laparatomy at the upper ends of one uterine horn, through a hole made in the wall of the uterus.

Gestation was assessed by ultrasonography around day 25 postestrus (18). Then, at the time of farrowing, the number of piglets born was recorded. Survival rate at farrowing was the ratio of the number of live-born piglets to the number of vitrified/warmed blastocysts transferred and is expressed as percentage.

Data Collection and Statistical Analysis

The number of corpora lutea, the number of embryos collected, the number of vitrified morulae and/or blastocysts, and the number of embryos hatched after *in vitro* culture were recorded for each donor. The ovulation rate and the number of embryos collected were analyzed using the GLM procedure of the SAS program (28). The interactions between treatments and genotypes on the hatching rate were analyzed by the CATMOD procedure of the SAS program (28). Pairwise comparison was made with the S-PLUS proportion test (30). Since a constant number of blastocysts was transferred to

TABLE 2
In Vitro Hatching Rate for Large White Hyperprolific and Meishan Blastocysts According to Vitrification Medium

Medium	Large White hyperprolific	Meishan
TCM	41%[c,d] (46) [7]	43%[b,d] (48) [7]
PBS	27%[a,c] (45) [6]	67%[a,b,f] (48) [6]
Control (nonvitrified)	70%[e] (40) [13]	72%[e,f] (11) [4]

Note. (n), Number of embryos; [*n*], number of replicates.
[a] $P = 0.001$; [b] $P = 0.02$; [c] $P = 0.21$; [d] $P = 0.97$; [e] $P = 0.98$; [f] $P = 0.98$.

recipients, the number of piglets born (the survival rate) was analyzed with the Wilcoxon rank sum test; the number of piglets was set to zero when recipient was open.

RESULTS

Embryo Production and Collection

The ovulation rate was significantly higher ($P = 0.0001$) in Large White hyperprolific gilts (17.4 ± 0.7; mean ± SE) than in Meishan gilts (14.5 ± 0.7). The number of embryos collected differed between genotypes: 12.9 ± 0.8 for Large White hyperprolific and 10.4 ± 0.8 for Meishan ($P < 0.01$). Average embryo collection rate was 74%, which was the ratio of the number of collected embryos to the number of corpora lutea.

Experiment 1: Effect of the Vitrification Medium and Genotype of Blastocysts on Hatching Rate after in Vitro Development

As shown in Table 2, a difference between genotypes was observed in hatching rates when PBS was used. The hatching rate for Meishan blastocysts was significantly higher than that for Large White hyperprolific blastocysts (67% vs 27%, respectively, $P = 0.001$) and not significantly different from that of the control Meishan blastocysts (67% vs 72%, respectively, $P = 0.98$) or from that of the control Large White hyperprolific blastocysts. For Meishan blastocysts, the hatching rate differed between PBS and TCM ($P = 0.02$). Results with TCM did not differ between the two genotypes. The hatching rate was 41 and 43% for Large White hyperprolific and Meishan, respectively ($P = 0.97$).

TABLE 3
In Vitro Hatching Rate for Large White Hyperprolific and Meishan Morulae

Treatments	Large White hyperprolific	Meishan
Vitrified/warmed	11.5%[a] (61) [9]	14%[a] (57) [9]
Control (nonvitrified)	64.5%[b] (17) [8]	72%[b] (11) [4]

Note. (n), Number of embryos; [*n*], number of replicates.
[a] $P = 0.41$; [a,b] $P = 0.0001$.

Experiment 2: Effect of Developmental Stage and Genotype

Vitrification at the morula stage was followed by a low *in vitro* survival rate, and there was no significant difference between the two genotypes (LWh 11% vs MS 14%; $P = 0.41$). These rates were significantly lower than those from the control group, both in Large White hyperprolific and in Meishan (Table 3; $P = 0.0001$).

Experiment 3: Viability of Vitrified/Warmed Meishan or Large White Hyperprolific Blastocysts after Transfer

Overall, the farrowing rate was 55% for the 20 transfers but this parameter varied between the two genotypes, 80% in Meishan and 30% in Large White hyperprolific (Table 4), and the difference was significant ($P = 0.04$). Five of

TABLE 4
Results of Farrowings Obtained after Transfer (20 Vitrified/Warmed Blastocysts per Recipient)

Genotypes	Number of recipient gilts	Number of farrowed gilts	Farrowing rate
Large White hyperprolific	10 [9]	3	30%[a]
Meishan	10 [9]	8	80%[b]
Total	20	11	55%

Note. [*n*], number of replicates.
[a,b] $P = 0.04$.

TABLE 5
Litter Size and Fetal Survival Rate after Transfer (20 Vitrified/Warmed Blastocysts per Recipient)

Genotypes	Number of piglets born per farrowing	Total number of live-born piglets	Total number of transferred blastocysts	Survival rate
Large White hyperprolific	5-5-2	11	200	5.5%[a] (11/200)
Meishan	4-4-4-5-5-4-2-1	27	200	13.5%[b] (27/200)

[a,b] $P = 0.04$.

10 recipients of Large White hyperprolific blastocysts became pregnant (Tables 4 and 6), and 3 gave birth to five, five, and two piglets (Table 5). The nonpregnant recipients returned to estrus within the normal expected period (19 days), with the exception of 2 that occurred at 29 and 39 days (Table 6). Ten transfers were performed with Meishan blastocysts, 9 recipients became pregnant, and 8 recipients farrowed from one to five piglets (Table 5). The 2 open recipients returned to estrus at days 20 and 27 postestrus (Table 6). Survival rate at farrowing was significantly higher in Meishan than in Large White hyperprolific: 13.5 and 5.5%, respectively ($P = 0.04$; Table 5). Piglets born after transfer were normal, and no anatomical abnormalities were observed after farrowing. Live-born piglets had birth and weaning weights within the normal range for these genotypes (data not shown).

DISCUSSION

The purpose of this study was to attempt to verify whether cooling rate was a key factor in porcine embryo vitrification. For species for which embryo vitrification is difficult, such as *Drosophila* spp. (31), an increase in cooling rate provided positive results. Faster cooling may be obtained by using a smaller volume of cryoprotectant to hold the embryos and by reducing the thickness of the straw wall, allowing closer contact with the liquid nitrogen. OPS technology provides both of these elements, which increased cooling rate approximately 10-fold compared to those achieved in standard straws (34). Vajta *et al.* (35) and Holm *et al.* (11) obtained higher *in vitro* hatching rates (73 and 71%, respectively) but only with Landrace embryos. *In vivo,* Beebe *et al.* (1) obtained piglets after centrifugation, cytoskeletal stabilization, vitrification, and transfer of early blastocysts.

TABLE 6
Individual Data of Return in Estrus for Open Recipients ($n = 9$)

Genotypes	Return in estrus <20 days	Return in estrus >21 days
Large White hyperprolific	20-19-20-18-19	29*–39*
Meishan	20	27*

* These recipients were controlled pregnant at day 25 of gestation by ultrasonography.

Our studies indicated that other factors may also influence *in vitro* survival after vitrification. The developmental stage of the embryo at the time of vitrification seems to be important. Despite an increased cooling speed, the hatching rate after *in vitro* development for morulae remained low (Table 3) and confirmed previous studies showing that cryoconservation of pig embryos can be performed successfully either at hatched blastocyst stages (6) or by mixing blastocysts and hatched blastocysts (13). However, it is not established whether or not *in vitro* development of morulae is fully efficient (7, 9, 22).

Dilution media of cryoprotectants may also play an important role in cryoinjuries and survival. The hatching percentage of blastocysts vitrified/warmed with PBS was significantly lower than that with TCM for the Large White

hyperprolific genotype. TCM199 medium contains many amino acids, which may act protectively, and the Hepes-buffered system, which may also have a favorable effect on Large White hyperprolific embryos. It has recently been shown that, for hamster embryos, cryopreservation reduces the ability to regulate intracellular pH (14). The PBS consisted of a phosphate buffer whose osmolarity was increased with a PBS × 2 concentration to compensate for the salt dilution resulting from the addition of a high concentration of cryoprotectants. The low *in vitro* survival rates (27%) obtained with this medium for the Large White hyperprolific genotype may be explained by the negative effect of the high concentration of NaCl (29), which is not detrimental for the Meishan. For Meishan blastocysts, the PBS medium resulted in much better survival rates, nearly identical with that of the control group: 67% vs 72% (Table 2).

For *in vivo* study, PBS was chosen because that medium was much better for the Meishan blastocysts; it has allowed us to obtain high farrowing rates compared to those of Large White hyperprolific (80% vs 30%). This point again emphasizes that embryo genotype is an important factor.

An *in vivo* study with 180 early blastocysts treated with cytochalasin, centrifuged, vitrified with OPS, and transferred resulted in five piglets being born (1). Survival rate was low (3%) and may be explained by the number of manipulations, by different experimental conditions, and/or by different genotypes.

Another experiment with OPS, conducted with an experimental outline similar to ours (11), did not result in piglets being born after transfer. However, several differences may have influenced these results:

- The time of equilibration in Cryoprotectant 2 was different: 1 min in our method vs 0.5 min in their method. The extended time in our study may allow better penetration of cryoprotectants.
- The genotypes of donors and recipients were different: Holm *et al.* (11) used Danish Landrace sows and Large White crossbred gilts. An *in vivo* and *in vitro* difference between Meishan and Large White hyperprolific blastocysts has been observed after vitrification. Moreover, we have shown that embryonic development varies between Large White and Meishan (32). Furthermore, the genotype of the recipient can affect the efficiency of embryo transfer; a higher pregnancy rate was obtained with transfer to Meishan recipients (83%; n = 48) than with transfer to crossbred recipients (54%; n = 48) (20).
- The transfer time in our study was with the same time lag; recipient gilts were asynchronous (−24 h) compared to donors. In Holm *et al.* (11), asynchrony is difficult to evaluate because embryos were cultured *in vitro* 1 to 3 days before vitrification. Pregnancy rate was always higher when transfers were made to recipients in which the onset of estrus was either synchronous with that of donors or 1 to 2 days later (25).

Survival rate at farrowing was low for both genotypes, 13.5% in Meishan and 5.5% in Large White hyperprolific, but these results were in agreement with other reports for hatched blastocysts. Dobrinsky *et al.* (9) obtained 6% (n = 157) and 13% (n = 224) of piglets after transfer of cytoskeletal-stabilized vitrified hatched blastocysts, and Kobayashi *et al.* (13) obtained 6.3% (n = 64) after transfer of vitrified expanded and hatched embryos.

An increase in this survival percentage may be obtained by reducing the number of transferred embryos, as suggested, after the transfer of fresh embryos (4, 25). Most of the recipients in these studies were European breeds, and they received 11 or more unhatched embryos. The percentages varied between 26 and 65% (4, 25). Cameron *et al.* (4) concluded that the optimum number of embryos that should be transfered to each recipient was between 12 and 16. For Meishan gilts, the optimum ranged around 10 embryos per recipient (17).

CONCLUSIONS

The cooling and/or warming rate is a key factor in the preservation of porcine embryos.

Genotype also seems to be an important factor, since embryonic development is not the same among genotypes. The stage of development is also important, and our method was found to be uniquely efficient for cryopreservation of blastocysts *in vitro*.

The open pulled straw technology is a reliable and efficient method for cryopreservation of porcine unhatched blastocysts. High pregnancy and farrowing rates can be achieved, opening a new possibility for the porcine embryo transfer industry. Moreover, the method is simple and easy to implement. It may become a new tool for conservation of genetic resources.

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REFERENCES

1. Beebe, L. F. S., Cameron, R. D. A., Blackshaw, A. W., Higgins, A., and Nottle, M. B. Piglets born from vitrified zona-intact blastocysts. *Theriogenology* **53,** 249 (2000).
2. Berthelot, F., Martinat-Botté, F., Locatelli, A., and Terqui, M. Cryoconservation d'embryons porcins âgés de 5 à 6 jours en utilisant une méthode de refroidissement ultra-rapide: la Méthode Open Pulled Straw (OPS). *J. Recherche Porcine France* **32,** 433–437 (2000).
3. Berthelot, F., and Terqui, M. Effects of oxygen, CO_2, pH and medium on in vitro development of individually cultured porcine one- and two-cell embryos. *Reprod. Nutr. Dev.* **36,** 241–251 (1996).
4. Cameron, R. D. A., Durack, M., Fogarty, R., Putra, D. K. H., and McVeigh, J. Practical experience with commercial embryo transfer in pigs. *Aust. Vet. J.* **66,** 314–318 (1989).
5. Després, P., Martinat-Botté, F., Lagant, H., Terqui, M., and Legault, C. Comparaison des performances de reproduction des truies appartenant à trois génotypes: Large-White (LW), Large-White "hyperprolifique" (LWh), Meishan (MS). *J. Recherche Porcine France* **24,** 345–350 (1992).
6. Dobrinsky, J. R. Cellular approach to cryopreservation of embryos. *Theriogenology* **45,** 17–26 (1996).
7. Dobrinsky, J. R. Cryopreservation of pig embryos. *J. Reprod. Fertil. Suppl.* **52,** 301–312 (1997).
8. Dobrinsky, J. R., Pursel, V. G., Long, C. R., and Johnson, L. A. Birth of normal piglets after cytoskeletal stabilisation of embryos and cryoconservation by vitrification. *Theriogenology* **49,** 166 (1998).
9. Dobrinsky, J. R., Pursel, V. G., Long, C. R., and Johnson, L. A. Birth of piglets after transfer of embryos cryopreserved by cytoskeletal stabilization and vitrification. *Biol. Reprod.* **62,** 564–570 (2000).
10. Haley, C. S., Ashworth, C. J., Lee, G. J., Wilmut, I., Aitken, R. P., and Ritchie, W. British studies of the genetics of prolificacy in the Meishan pig. *In* "Chinese Pig Symposium" (M. Molenat and C. Legault, Eds.), pp. 85–97. INRA, Paris, 1990.
11. Holm, P., Vajta, G., Machaty, Z., Schmidt, M., Prather, R. S., Greve, T., and Callesen, H. Open Pulled Straw (OPS) vitrification of porcine blastocysts: Simple procedure yielding excellent in vitro survival, but so far no piglets following transfer. *Cryo–Letters* **20,** 307–310 (1999).
12. Kobayashi, S., Goto, M., Kano, M., Takei, M., Minato, K., and Leibo, S. P. Farrows or pregnancies by transfer of porcine embryos vitrified at two institutions. *Cryobiology* **37,** 436 (1998).
13. Kobayashi, S., Takei, M., Kano, M., Tomita, M., and Leibo, S. P. Piglets produced by transfer of vitrified porcine embryos after stepwise dilution of cryoprotectants. *Cryobiology* **36,** 20–31 (1998).
14. Lane, M., Lyons, E. A., and Bavister, B. D. Cryopreservation reduces the ability of hamster 2-cell embryos to regulate intracellular pH. *Human Reprod.* **15,** 389–394 (2000).
15. Le Gal, F., Deroover, R., Verhaeghe, B., Etienne, D., and Massip, A. Birth of calves from vitrified oocytes. *Ann. Med. Vet.* **144,** 33–36 (2000).
16. Martinat-Botté, F., Plat, M., Procureur, R., Després, P., and Locatelli, A. Importance du génotype de la donneuse et de la receveuse pour la production et le transfert d'embryons chez la truie. *J. Recherche Porcine France* **24,** 315–320 (1992).
17. Martinat-Botté, F., Plat, M., Procureur, R., and Terqui, M. Meishan (MS) as recipient in embryo transfer programs. *Int. Symp. Chinese Pig Breeds Harbin China.* pp. 561–564 (1992).
18. Martinat-Botté, F., Renaud, G., Madec, F., Costiou, P., and Terqui, M. "Ultrasonography and Reproduction in Swine: Principles and Practical Applications." INRA Editions & Intervet, Paris, 2000.
19. Martino, A., Songsasen, N., and Leibo, S. P. Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling. *Biol. Reprod.* **54,** 1059–1069 (1996).
20. Miwa, H., Fujii, J., Kanno, H., Taniguchi, N., and Aozasa, K. Pyruvate secreted by human lymphoid cell lines protects cells from hydrogen peroxide mediated cell death. *Free Radical Res.* **33,** 45–56 (2000).
21. Mödl, J., Reichenbach, H.-D., Wolf, E., and Brem, G. Development of frozen–thawed porcine blastocysts in vitro and in vivo. *Vet. Rec.* **139,** 208–210 (1996).

22. Nagashima, H., Kashiwasaki, N., Ashman, R., Grupen, C., Seamark, R. F., and Nottle, M. Recent advances in cryopreservation of porcine embryos. *Theriogenology* **41,** 113–118 (1994).
23. Nagashima, H., Kashiwazaki, N., Ashman, R. J., Grupen, C. G., and Nottle, M. B. Cryopreservation of porcine embryos. *Nature* **374,** 416 (1995).
24. Petters, R. M., and Wells, K. D. Culture of pig embryos. *J. Reprod. Fertil. Suppl.* **48,** 61–73 (1993).
25. Polge, C. Embryo transplantation and preservation. *In* "Control of Pig Reproduction" (D. J. A. Cole and G. R. Foxcroft, Eds.), pp. 277–291. Butterworth Scientific, London, 1982.
26. Pollard, J. W., and Leibo, S. P. Chilling sensitivity of mammalian embryos. *Theriogenology* **41,** 101–106 (1994).
27. Rall, W. F. Factors affecting the survival of mouse embryos cryopreserved by vitrification. *Cryobiology* **24,** 387–402 (1987).
28. SAS Institute Inc. SAS STAT Guide for personnal computers, version 6.12. Cary, NC, 1997.
29. Stachecki, J. J., Cohen, J., and Willadsen, S. Detrimental effects of sodium during mouse oocyte cryopreservation. *Biol. Reprod.* **59,** 395–400 (1998).
30. Statistical Science. S-PLUS Guide to statistical and mathematical analysis, version 3.4. Seattle, StatSci, a division of MathSoft, Inc., 1996.
31. Steponkus, P. L., Myers, S. P., Lynch, D. V., Gardner, L., Bronshteyn, V., Leibo, S. P., Rall, W. F., Pitt, R. E., Lin, T. T., and MacIntyre, R. J. Cryopreservation of *Drosophila melanogaster* embryos. *Nature* **345,** 170–172 (1990).
32. Terqui, M., Bazer, F. W., and Martinat-Botté, F. Mechanisms of high embryo survival in Meishan gilts. *Int. Symp. Chinese Pig Breeds Harbin China.* pp. 52–58 (1992).
33. Vajta, G., Booth, P. J., Holm, P., Greve, T., and Callesen, H. Successful vitrification of early stage bovine *in vitro* produced embryos with Open Pulled Straw (OPS) method. *Cryo-Letters* **18,** 191–195 (1997).
34. Vajta, G., Holm, P., Booth, P. J., Jacobson, H., Greve, T., and Callesen, H. Open Pulled Straw (OPS) vitrification: A new way to reduce cryoinjuries of bovine ova and embryos. *Mol. Reprod. Dev.* **51,** 53–58 (1998).
35. Vajta, G., Holm, P., Greve, T., and Callessen, H. Vitrification of porcine embryos using the Open Pulled Straw (OPS) method. *Acta Vet. Scand.* **38,** 349–352 (1997).

附件四

MELATONIN

Biology of Mammalian Photoperiodism and the Critical Role of the Pineal Gland and Melatonin

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Abstract In mammals, photoperiodic information is transformed into a melatonin secretory rhythm in the pineal gland (high levels at night, low levels during the day). Melatonin exerts its effects in discrete hypothalamic areas, most likely through MT1 melatonin receptors. Whether melatonin is brought to the hypothalamus from the cerebrospinal fluid or the blood is still unclear. The final action of this indoleamine at the level of the central nervous system is a modulation of GnRH secretion but it does not act directly on GnRH neurones; rather, its action involves a complex neural circuit of interneurones that includes at least dopaminergic, serotoninergic and aminoacidergic neurones. In addition, this network appears to undergo morphological changes between seasons.

Key words melatonin, photoperiod, mammals, rhythm, reproduction

To cope with seasonal fluctuations in environmental conditions, particularly temperature and food availability, most long-lived species of mammal exhibit seasonal cycles of physiological functions and morphological changes (see Goldman, 2001 [this issue]). The main adaptive mechanism widely observed in the wild is the ability to restrict breeding activity to the time of the year that coincides with the most propitious conditions for the survival of the neonates. This article will provide an overview of how photoperiod controls seasonal variations in reproductive activity, with a focus on the role and mode of action of melatonin.

PHOTOPERIOD AS THE MAIN CUE FOR SEASONAL TIMING

The use of photoperiod as a predictive cue was first demonstrated in a mammal by Baker and Ranson (1932). They observed that in a colony of field voles held on 15 h of light, reproduction occurred. However, when exposed to 9 h of light per day, reproduction was blocked. Since then, photoperiodism has been shown to be the strongest synchronizer of seasonal functions in most mammalian orders (for review, see Goldman, 2001). In Djungarian hamsters, exposure to short days induces reproductive inhibition, winter molt, onset of daily torpor, and increase in body mass, while maintenance of animals in long days prevents these changes. In sheep, the reversal of the annual photoperiodic cycle, without any modification of other environmental factors, causes the breeding season to phase shift by 6 months; the reduction of its period to 6 months induces the appearance of two periods of reproductive activity every year (for review, see Malpaux et al., 1993a).

The response to photoperiod in mammals is not based solely on absolute day length, but also on photoperiodic history of the animals. In the ewe, 13 h of light per day cause a stimulation of luteinizing hormone (LH) secretion if animals have been exposed

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previously to 16 h of light. In contrast, 13 h of light per day inhibit LH secretion if animals had been exposed previously to 10 h of light. Thus, exposure to a same photoperiod may produce opposite effects depending on the photoperiodic history of the animals (Robinson and Karsch, 1988). Similar results have been obtained in several other species, and the importance of photoperiodic history appears to be a general characteristic of photoperiodic responses (Gorman and Zucker, 1998; Goldman, 2001).

In many species, seasonal reproductive transitions are timed by photoperiodic cues through synchronization of an internally generated rhythm, called a circannual rhythm because its period approximates one year (Karsch et al., 1989). How photoperiod entrains a circannual rhythm has been well studied in the case of reproductive activity in the ewe. This animal normally enters the breeding season in the autumn and, in the absence of pregnancy, has regular ovulatory cycles until mid-winter. Ovulations then cease, and the ewe remains anovulatory throughout the summer (Karsch et al., 1984). When these animals are deprived of information about time of year, either by being kept in constant photoperiod or by blinding, they continue in most cases to show long-term changes in ovulatory activity or in LH secretion (Karsch et al., 1989; Jansen and Jackson, 1993). Similar observations were made on testicular size changes in rams (Howles et al., 1982). If ewes are kept for 5 years in constant short photoperiod (8L:16D), a cycle of LH secretion is observed with a period shorter than a year and cycles are desynchronous among individuals and out of phase with respect to the cycles of sheep maintained outdoors (Karsch et al., 1989). Interestingly, it is possible to render ewes incapable of perceiving photoperiod by pinealectomy and then to infuse specific circadian patterns of melatonin to restore *photoperiodic information* (see below). Such studies indicate that the entire annual photoperiodic cycle is not required for the entrainment of the circannual rhythm of LH secretion; therefore, synchronization of annual cycles do not require continuous input from the environment (Fig. 1). Not all cues are equally effective at synchronization; in this animal, the increasing day length of winter and spring is critically important to impose a 365-day period to the rhythm (Woodfill et al., 1994; Barrell et al., 2000). A similar timing mechanism is observed in hibernating circannual species. For instance, in ground squirrels and woodchucks, photoperiodic exposure in spring/summer could maintain precisely timed seasonal cycles despite the disruption of photoperiodic signaling during periods of hibernation in autumn/winter (Zucker, 1985; Concannon et al., 1997).

Other species, such as many short-lived rodents, do not seem to display circannual cycles as they remain reproductively active as long as they are maintained in long photoperiod (Pévet, 1988). The Syrian hamster requires short days to induce gonadal regression at the end of the breeding season. Subsequent gonadal recrudescence that occurs in midwinter does not require an active photoperiodic drive; the transition into breeding season is generated by the development of refractoriness to the short days of winter, which allows this process to occur when days are still short. For the appropriate response to the decreasing day lengths of the following autumn to occur, the state of photoresponsiveness must be restored. This is accomplished only if the individual experiences several weeks of exposure to long days, as occurs each summer in the wild. Although the development of refractoriness is a form of endogenous timekeeping, the requirement for short days to inhibit reproductive activity and that for long days to restore photoresponsiveness make hamsters unable to exhibit circannual cycle of reproductive activity when they are exposed chronically to either short or long days (Goldman and Nelson, 1993).

ROLE OF THE PINEAL GLAND AND MELATONIN IN TRANSDUCING PHOTOPERIODIC INFORMATION

The importance of the pineal gland was demonstrated by numerous experiments showing that the effect of photoperiod on seasonal functions is profoundly altered in pinealectomized animals. In Syrian hamsters, pinealectomy prevents the seasonal reduction in gonadotropin secretion and the gonadal regression normally brought about by experimental or natural short photoperiod. Injection of melatonin reverses the effect of pinealectomy on gonadotropin secretion and causes gonadal regression (for review, see Pévet, 1988). This early work, mainly in hamsters, led to the concept of the antigonadal action of melatonin. However, more recent data led to the more generalized concept of a role for melatonin in the control of seasonal rhythms. Specifically, pinealectomy or superior cervical ganglionectomy suppresses responses to both

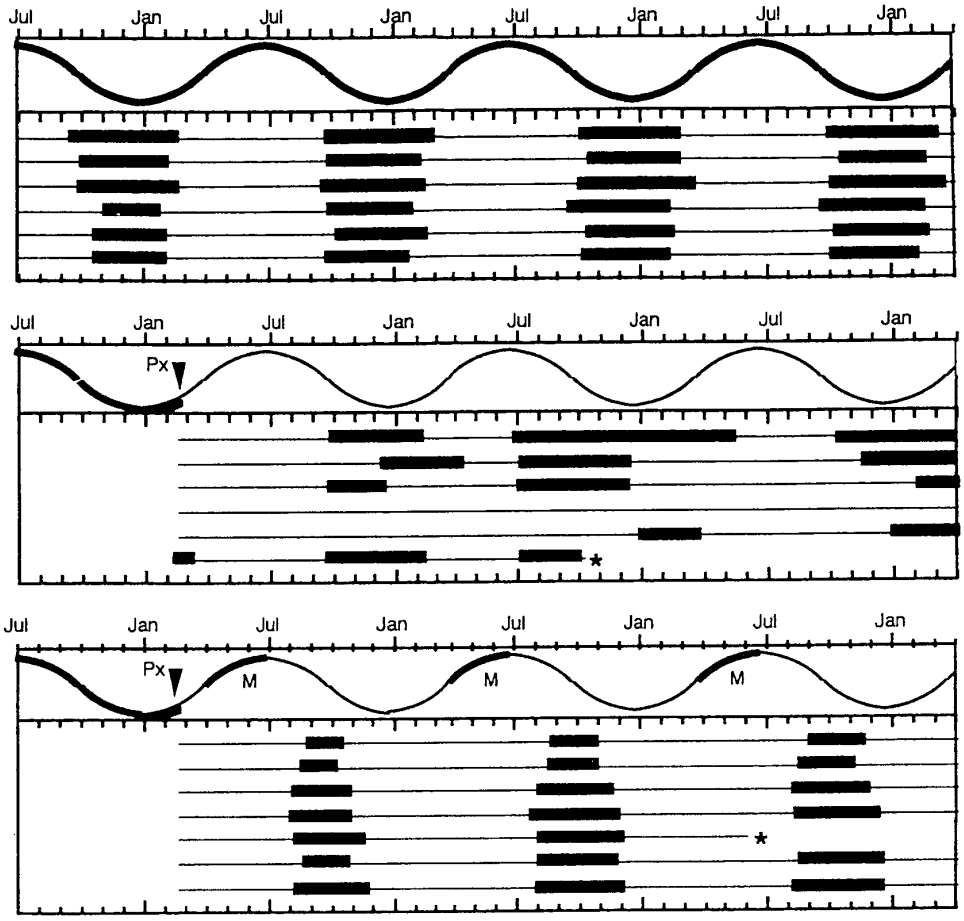

Figure 1. Synchronization of the annual cycle of reproduction in the ewe. Top: annual cycle of luteinizing hormone (LH) secretion in pineal-intact ewes (black bars represent the periods of elevated LH secretion indicative of high reproductive neuroendocrine activity in the model of ovariectomized ewes treated with estradiol used in this experiment). Middle: circannual changes in LH secretion in pinealectomized ewes deprived of photoperiodic information (changes are not synchronous among ewes and are desynchronized from the natural changes represented in top panel). Bottom: entrained changes in LH secretion in pinealectomized, which receive *photoperiodic information* by infusion of melatonin during the spring only (heavy photoperiodic curve). This 90-day period of information is sufficient to synchronize the rhythms of the ewes. Arrow represents the time of pinealectomy and stars the time of death of 2 animals (Adapted from Woodfill et al., 1994).

short and long photoperiod, and melatonin, depending on its specific pattern, reinstates both these responses (Fig. 2; Lincoln and Short, 1980; Carter and Goldman, 1983; Karsch et al., 1984). This role was well illustrated by replacement studies in pinealectomized animals. Melatonin delivered into the peripheral circulation to mimic short-day or long-day profiles can reproduce the stimulatory effects of short days or the inhibitory actions of long days on LH secretion in the ewe (Karsch et al., 1984). Similar conclusions for a role of melatonin were obtained concerning other physiological seasonal functions such as molt and pelage changes, prolactin secretion, thermoregulation, hibernation, and body mass changes (Bartness et al., 1993).

Pineal melatonin secretion is regulated by light through a multistep nervous pathway connecting the retina to the pineal gland, including the suprachiasmatic nuclei as a major step (see Schwartz et al., 2001 [this issue] for review). The secretion is exclusively nocturnal, but in some species (sheep, etc.), it is elevated throughout the dark period, whereas in others (Syrian hamster, rat, etc.), melatonin secretion does not begin until a few hours after the onset of darkness (Arendt, 1995). The amplitude of the melatonin rhythm (difference between nighttime and daytime levels) is highly variable among animals, but it is highly repeatable within individuals with a strong genetic determinism (Zarazaga et al., 1998). It is

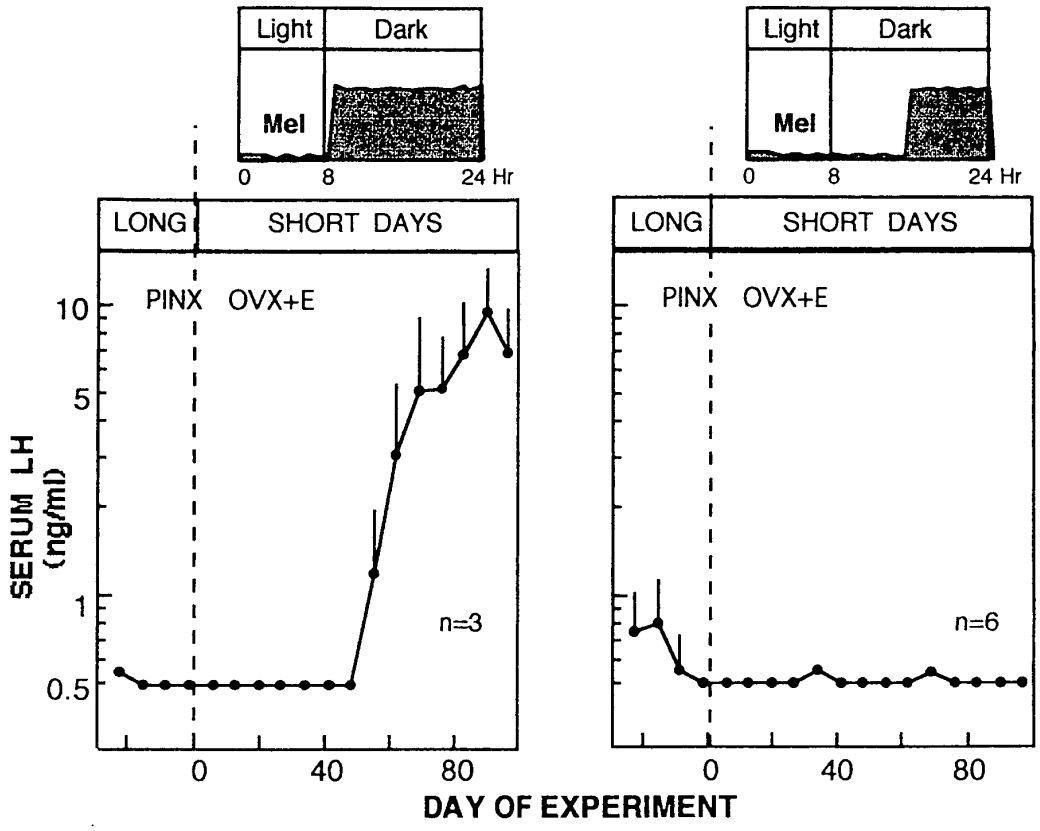

Figure 2. Serum luteinizing hormone (LH) concentrations in pinealectomized (PINX) ovariectomized and estradiol-treated (OVX + E) ewes in which photoperiod and melatonin (Mel) were either matched (left, 16 h of melatonin infusion in short days of 8L:16D) or mismatched (right, 8 h of melatonin in short days of 8L:16D) beginning on Day 0. Before day 0, melatonin was infused for 8 h per day (long-day profile) during long days (16L:8D) to suppress LH values and sensitize ewes to the stimulatory effects of short-day melatonin. LH values are presented as weekly means of twice weekly samples; *n* indicates the number of ewes. The light-dark cycle and pattern of melatonin infused after Day 0 are shown by the key at the top. (Adapted from Karsch et al., 1988).

related to the size of the pineal gland and the number of pinealocytes that it contains (Coon et al., 1999). The main characteristic of the melatonin secretory rhythm that conveys the photoperiodic information appears to be the duration of secretion (length of time with elevated levels). Indeed, when physiological doses of melatonin are infused into pinealectomized Djungarian hamsters and sheep for fixed durations, but at different times relative to the light-dark cycle, the response varies only with the duration of the infusion, and not with time of day (Bartness et al., 1993). This critical role of duration is in keeping with the fact that the duration of melatonin secretion is positively correlated to the length of the night in all species regardless of the exact shape of the melatonin profile.

The definition of duration is not absolute, but relative. The interpretation of the duration of a given melatonin pattern depends on the photoperiodic and melatonin history of the animal. As discussed above, the direction of change is more important than the absolute duration of the photoperiod to determine a physiological response. A change in day length is always accompanied by a similar change in the duration of melatonin secretion (Robinson and Karsch, 1988).

Overall, response to the annual changes in day length requires a complex set of mechanisms to detect three types of changes: First, detection of the presence of melatonin above a minimum threshold (night vs. day); second, detection of the duration of presence of melatonin above this threshold (long vs. short days); and finally, detection of changes in the duration of melatonin presence relative to previous melatonin exposure (increasing vs. decreasing day length).

MODE OF ACTION OF MELATONIN FOR THE CONTROL OF GnRH RELEASE

Target Sites and Melatonin Receptors

Localization of Target Sites

The identification of the sites of action of melatonin is made difficult since melatonin influences many physiological functions (Arendt, 1995). Melatonin could either act at a single site in the brain or pituitary, which would then be implicated in the regulation of many seasonal functions, or melatonin could act at multiple sites, each involved in regulating one seasonal function. This difficulty is expanded by the localization of high-affinity melatonin receptors in a wide variety of tissues in the body (for review, see Bittman, 1993).

The first demonstration that melatonin acted in the brain was provided by studies in the white-footed mouse using small beeswax pellets impregnated with melatonin (Glass and Lynch, 1982). More evidence for a brain site was obtained with a rhythmic pattern of intracerebral melatonin delivery in pinealectomized white-footed mice (Dowell and Lynch, 1997) or in Syrian hamsters and gerbils (Hastings et al., 1988; Devries et al., 1989). However, these studies could not define a precise target within the brain.

The development of the melatonin probe, ^{125}I-melatonin allowed for the identification of putative target sites of melatonin within the hypothalamo-hypophyseal system, whose importance was then tested by functional studies, mainly in hamsters and sheep. Although binding was found in several areas within the brain and the pituitary (Bittman, 1993), it was the pars tuberalis (PT) that drew most attention since the density of binding was much higher than in any other hypothalamic or pituitary site, a consistent feature among species investigated to date. Surprisingly, studies in sheep and hamsters have led to the conclusion that the PT does not mediate the action of melatonin on the neuroendocrine reproductive axis. Indeed, in the ewe, melatonin delivered directly to the PT does not appear to modify the secretion of LH: Neither the placement of a melatonin microimplant directly against the anterior face of the PT nor the discrete insertion of a microimplant into the PT modified LH secretion (Malpaux et al., 1997). In contrast, microimplants placed in the MBH or third ventricle stimulated LH release (Malpaux et al., 1993b; Malpaux et al., 1997). These studies provide definitive evidence that the hypothalamus and not the PT is the important melatonin target for transducing the effects of this indoleamine on the reproductive neuroendocrine axis. Moreover, the placement of microimplants in the PT or pars distalis of Soay rams led to similar conclusions (Lincoln and Maeda, 1992). In this species, melatonin binding was found in the pre-mammillary hypothalamic area: It is located at the base of the brain and limited dorsally by the fornix; it extends 3 mm on either side of the third ventricle, is posterior to the infundibular recess, and is delimited caudally by the mammillary bodies (Fig. 3; Chabot et al., 1998; Malpaux et al., 1998). A clear relationship was observed between the proximity of melatonin microimplants to the area of binding and the effectiveness of these microimplants to stimulate LH, strongly suggesting that melatonin targets are located in the pre-mammillary hypothalamic (PMH) area (Malpaux et al., 1998). In addition, a strong contrast between a high *cfos* expression during the day and a low one at night is observed in this area (Daveau et al., 1999). This day/night change is pineal and melatonin dependent. Indeed, it is no longer expressed in pinealectomized animals, and short-day infusion of melatonin leads to a reduction of *cfos* expression in this structure. A similar day-night change in *cfos* expression is in the SCN but, in this structure, it is not dependent on the pineal gland (Malpaux et al., 2000). In Syrian hamsters, the hypothalamic localization of the target sites of melatonin for acting on reproduction was also strongly suggested. Lesions of the dorsomedial hypothalamus melatonin-binding sites block the gonadotropic response of male Syrian hamsters to short photoperiod or melatonin, and lesions of the ventromedial hypothalamus cause premature testicular recrudescence in inhibitory photoperiod (Maywood and Hastings, 1995; Bae et al., 1999). Furthermore, an overlap between melatonin binding and androgen receptor immunoreactivity is found in the dorsomedial nucleus, and it was suggested that sensitivity to steroid feedback, a key mechanism of the action of melatonin on gonadotropin secretion, might be influenced in this area in Syrian hamsters (Maywood et al., 1996). Although the phenotype of the target cells remains to be established, these data in hamsters and sheep suggest a discrete target for melatonin in the hypothalamus.

The PT mediates, at least in part, the action of melatonin on prolactin secretion (Lincoln and Clarke,

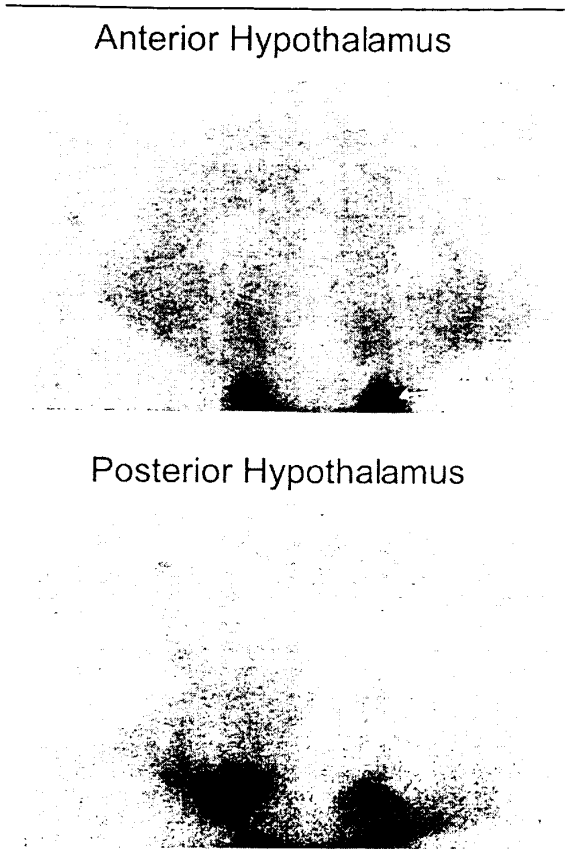

Figure 3. Coronal sections of sheep hypothalamus illustrating distribution of melatonin binding sites. Top: section in the anterior hypothalamus. Note the high level of binding in the pars tuberalis (PT). Bottom: section in the posterior hypothalamus. Note the presence of binding in the premammillary area (base of the brain, limited dorsally by the fornix (fx) and on either side of the third ventricle (v)). (Adapted from Malpaux et al., 1998.)

1994; Malpaux et al., 1995). The dual sites of action of melatonin for controlling gonadotropin and prolactin secretion, respectively, are interesting in relation to the photoperiodic regulation of these two functions. Indeed, the action of photoperiod and melatonin on reproductive activity is characterized by long latency and the importance of photoperiodic history, which is consistent with an action at the level of the brain for the storage of information. In contrast, photoperiodic history is not critical to the regulation of prolactin secretion (Hastings et al., 1989), and the action of melatonin is relatively rapid (only a few days; Malpaux et al., 1997), which is consistent with a more "classical" endocrine regulation at the level of the pituitary, involving the tuberalin as a messenger (see Hazlerigg et al., 2001 [this issue]). More generally, given the diversity of localization of melatonin binding sites in the brain, it is possible that melatonin acts in several sites to exert its various reproductive effects (gonadotropin secretion, gonadal activities, sexual behavior, maternal behavior, etc.).

Type of Melatonin Receptor Mediating the Seasonal Effects of Melatonin

Cloning studies have identified three high-affinity melatonin receptor subtypes so far classified as MT1, MT2 (previously known as Mel1a and Mel1b, respectively), and Mel1c (Reppert et al., 1996). The three receptor subtypes MT1, MT2, and Mel1c are expressed in lower vertebrates. Both MT1 and MT2 are found (Reppert et al., 1996) in most mammalian species, although in some of them like sheep only MT1 has been shown to be expressed. All these subtypes display similar high binding affinity for melatonin and the same rank of order for the binding of common ligands (Dubocovich et al., 1995). A fourth melatonin receptor subtype, MT3, displaying lower affinity for melatonin (in the nanomolar range) was recently purified from human tissues using a biochemical approach and identified as the quinone reductase 2 (QR2) (Nosjean et al., 2000).

Natural knockout of the MT2 receptor gene in Djungarian hamsters does not alter seasonal reproductive and circadian responses (Weaver et al., 1996). Moreover, in mice the targeted disruption of MT1 showed that this receptor subtype is involved in the regulation of the circadian rhythms generated by the master clock located in the SCN (Liu et al., 1997). All together, these data suggest that MT1 mediates major biological functions of melatonin in mammals and, especially, the control of reproduction. Polymorphism has been detected for the human and ovine MT1 and the Xenopus Mel1c receptor subtypes (Brydon et al., 1999a). Interestingly, a correlation has been observed between the frequency of an allele of the gene of the MT1 receptor and the intensity of seasonal anovulatory activity in ewes (Fig. 4; Pelletier et al., 2000), which constitutes an additional indirect evidence for the implication of the MT1 subtype in the seasonal regulation of reproduction. Structurally, high-affinity melatonin receptor subtypes define a distinct receptor family within the superfamily of G-protein coupled receptor. Recent studies have shown that MT1 are functionally coupled to both PTX-sensitive and PTX-insensitive G proteins (Brydon et al., 1999b).

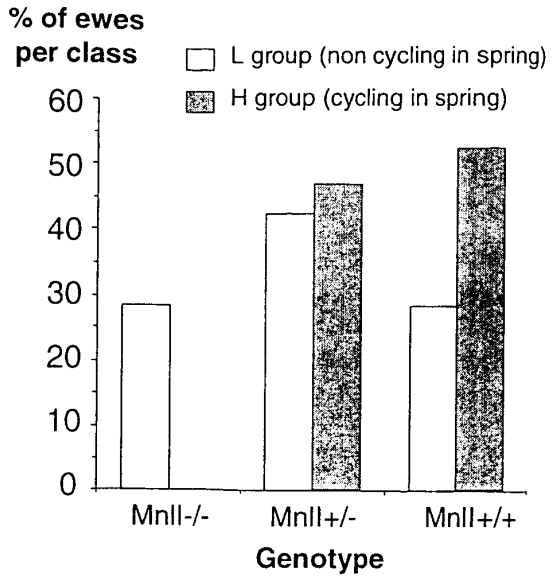

Figure 4. Distribution of Merinos d'Arles ewes according to their genotype (presence or absence of the polymorphic site MnlI of the melatonin receptor MT1). Ewes were selected according to their ability to display ovulatory cycle in the spring (group H, weak seasonal variations) or not (group L, strong seasonal variations). The frequency distribution of genotypes is different for the two groups (from Pelletier et al., 2000).

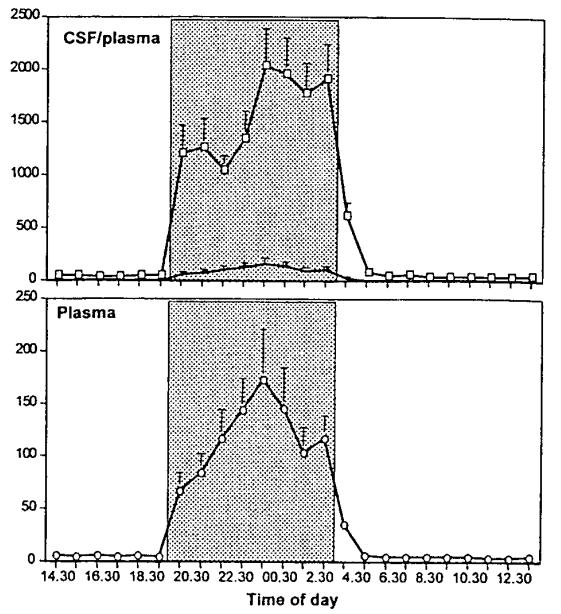

Figure 5. Profiles of melatonin concentrations in the jugular blood (top and bottom panels, open circle) or in the third ventricle cerebrospinal fluid (CSF) (top panel, open square). Scale is expanded on the bottom panel to visualize the shape of the melatonin profile in plasma (same curve as above). Note that levels are twentyfold higher in the CSF and coding for duration of melatonin presence is similar in the two compartments. Dark period is represented by shaded area (adapted from Skinner and Malpaux, 1999).

Route Used by Melatonin to Reach Its Hypothalamic Targets

Melatonin can use two possible routes to reach its central target sites. It may reach the hypothalamus through a circuitous peripheral pathway (pineal-jugular-carotid-target). Alternatively, melatonin could access the hypothalamus more directly through the third ventricle (pineal-IIIV-target). Several pieces of evidence are in favor of this latter hypothesis. First, the localization of the melatonin-binding sites of the PMH adjacent to the third ventricle makes diffusion of this small and highly lipophilic molecule from the cerebrospinal fluid (CSF) to the target sites likely (Malpaux et al., 1998). Second, melatonin levels are higher in the crebrospinal fluid than in the blood in several species such as sheep (Shaw et al., 1989) or goats (Kanematsu et al., 1989). Interestingly, melatonin levels at the base of the IIIV, near the PMH, are about 20 times as high as in jugular blood (Fig. 5) and 100 times as high as in carotid blood, making the CSF signal much stronger than the blood-borne one; importantly, the dusk increase and dawn decrease in melatonin levels are as sharp in both compartments, thus the coding for duration is equally good, a critical prerequisite if it is to play a role in transducing photoperiodic information (Skinner and Malpaux, 1999; Malpaux et al., unpublished). It is worth noting that IIIV melatonin most likely originates directly from CSF-contacting pinealocytes in the pineal recess since melatonin levels in this recess are much higher than in the rest of the ventricle (Tricoire et al., 2000; Hewing, 1980). This CSF hypothesis may appear at odds with studies showing that melatonin administered systematically to pinealectomized animals can entrain reproductive function (Karsch et al., 1984). These latter studies showed unequivocally that melatonin was the primary transducer of photoperiodic information, but no extrapolation about the route used by melatonin to get from its source to its target is possible. This is because the levels produced in these melatonin replacement studies replicated jugular concentrations and not carotid artery levels, which are about fivefold lower (Malpaux et al., unpublished).

Interneurones between Melatonin Target and GnRH Neurones

The variation in the duration of melatonin presence is processed neurally, and then the signal regulates GnRH secretion. For instance, in sheep, melatonin, given as a short-day profile, causes an increase in the frequency of pulsatile GnRH release (Malpaux et al., 1997). This increase in GnRH secretion is observed after a long lag time, that is, 40 to 60 days, when LH secretion is stimulated. These changes in GnRH secretion are the consequences of two complementary mechanisms controlled by melatonin: a direct steroid-independent modulation of GnRH secretion and a change in the steroid negative feedback on GnRH secretion (Goodman, 1994). Despite this action of melatonin via changes in GnRH secretion, several pieces of evidence indicate that melatonin does not act directly on GnRH neurones. First, GnRH immunoreactivity does not overlap with melatonin-binding sites' expression pattern (Lehman et al., 1997; Malpaux et al., 1998). Second, several neurotransmitter systems have been shown to be involved in the regulation of LH secretion by melatonin.

Dopamine

The role of dopamine in the regulation of GnRH secretion is well documented, and numerous pharmacological and anatomical data as well as surgical disruption of dopaminergic structures (Thiéry et al., 1989; Goodman, 1994) suggest that dopaminergic neurons could transduce the inhibitory effects of estradiol on GnRH release during the anestrous season in the ewe (Thiéry et al., 1995). This effect involves a dopaminergic cell group (A15) that is stimulated by estradiol and is responsible for the inhibition of GnRH secretion during seasonal anestrus (Goodman, 1994; Thiéry et al., 1995).

Independently of estradiol, photoperiod, via melatonin, modulates the activity of tyrosine hydroxylase (TH) in the dopaminergic terminals present in the median eminence in sheep (Malpaux et al., 1997). Specifically, exposure to short days or treatment with short-day-like melatonin causes a reduction in TH activity. This change is important in relation to the regulation of GnRH since blockade of the synthesis or the action of dopamine in this structure leads to a stimulation of LH secretion when it is applied on long-day-inhibited ewes (Bertrand et al., 1998). Therefore, exposure to short days causes a reduction in dopaminergic activity in the median eminence and, because dopamine is inhibitory of GnRH secretion, this change is partly responsible for the stimulation of GnRH and LH secretion. The median eminence (ME) in sheep is a structure rich in dopaminergic terminals but contains no TH-immunoreactive perikarya (Tillet, 1995). DA fibers form synaptic contacts with neurons secreting GnRH (Kuljis and Advis, 1989), providing anatomical support for ME as the terminal step for the GnRH secretion regulation pathway. Interestingly, the pharmacological blockade of TH activity no longer causes a stimulation of LH secretion when it is applied after a longer exposure to long days (71 days; Bertrand et al., 1998). These data suggest, therefore, that an increase in TH activity in the ME is an important component of the inhibitory effect of long-day melatonin profile on GnRH output and that it is critical for the initial phase of the inhibition. This observation is consistent with the hypothesis that different neural pathways participate in photostimulation, photoinhibition, or specific stages of these conditions (Hileman et al., 1994). For instance, lesions in the anterior hypothalamic area of the ewe delay, but do not suppress, the inhibitory effect of a shift from long to short days (Hileman et al., 1994). However, such modulation of TH activity appears to be E independent because the photoperiod-induced changes in TH activity are similar in OVX and OVX+E ewes (Malpaux et al., 1997). Thus, in contrast to the A15 and possibly A14 dopaminergic nucleus, which are involved in the modulation of the E-negative feedback, the dopaminergic neurons of the ME appear to be involved upstream relative to the integration of the E signal. The localization of the cell bodies projecting their axons to the ME, and more generally the anatomical and functional relationship between the A14 and A15 nuclei and the ME, have yet to be determined.

In male Syrian hamsters, exposure to inhibitory short days, presumably through melatonin secretion, reduces dopamine and noradrenaline turnover in the ME (Steger et al., 1995). This change is associated with a decrease in L-aromatic amino acid decarboxylase positive cells in the arcuate nucleus without changes in TH-immunoreactive cell number or TH activity (Krajnak and Nunez, 1996). Suppression of LH and follicle-stimulating hormone (FSH) release in this species is presumably related to reduced noradrenaline

activity, whereas reduced dopamine turnover may represent a consequence of suppression of prolactin levels by short days (Steger et al., 1995).

Serotonin

In the ewe, serotonin may play a role in the photoperiodic inhibition of GnRH secretion during seasonal anestrus (Goodman, 1994; Thiéry et al., 1995). This inhibitory effect is mediated by 5HT2A receptor subtypes, and photoperiod-induced changes in 5HT2A receptor density in the hypothalamus have been described (Le Corre et al., 1994; Pelletier et al., 1999).

Excitatory Amino Acids

When injected peripherally, an agonist for one of the glutamate receptor subtype, N-methyl-D,L-Aspartate (NMDA), increases LH secretion in species like sheep (Lincoln and Wu, 1991; Viguié et al., 1995) and hamsters (Ebling et al., 1995). The amplitude of the rise in LH secretion is more pronounced in photoinhibited than in photoactivated animals (Viguié et al., 1995). Interestingly, photoperiod was shown to modulate NMDA-specific binding in the hamster preoptic area (Urbanski and Pierce, 1991).

The implication of several neurotransmitter systems in the action of melatonin on GnRH neurones is strongly suggestive of the existence of a neuronal network connecting the target of melatonin to the GnRH system. Part of the mode of action of photoperiod could be to cause morphological changes in this neuronal network. Evidence for such an effect was obtained at the level of the GnRH neurons of the ewe. GnRH neurons in the preoptic area receive more than twice the mean number of synaptic inputs per unit of plasma membrane during the breeding season as during anestrus (Lehman et al., 1997). Also, NCAM, and its polysialylated form PSA-NCAM that promotes plasticity by modifying the stability of cell-cell contacts, is thought to be functionally significant to seasonal plasticity of the GnRH neurosecretory system. Indeed, in the Djungurian hamster, photoperiod-induced changes in gonadotropin secretion are associated to variations in the relative amounts of PSA and other NCAM isoforms in the basal hypothalamus (Lee et al., 1995). In sheep, the expression of PSA-NCAM is intimately associated with preoptic GnRH neurons, and this expression varies with the seasonal shift in reproductive neuroendocrine activity (Viguié et al., 2001). Interestingly, these changes are not dependent on changes in ovarian steroid levels, which differs markedly with the examples of seasonal plasticity described in the literature due to changing levels of steroids. The changes observed in the preoptic area of the ewe could, therefore, reflect the action of photoperiod and melatonin, or the expression of the circannual rhythm of reproduction, a key element of the photoperiod responsive mechanisms in this species (Karsch et al., 1995). In relation to possible phenomena of neuronal plasticity, it is worth noting that thyroid hormones have been strongly implicated in allowing seasonal changes in reproductive activity (Karsch et al., 1995). Since thyroid hormones are essential for the normal morphological maturation of the central nervous system (Granholm, 1985), the permissive role of thyroid hormones for seasonal changes to occur may be to cause the prerequired morphological rearrangements for the changes in GnRH secretion.

PRACTICAL ASPECTS OF THE CONTROL OF REPRODUCTION USING LIGHT, MELATONIN, AND GENETIC VARIABILITY

Overcoming seasonality of reproduction in species such as sheep or goats has drawn much interest to allow breeders to meet the constant demand of the consumers for their meat or milk. The basic principle to control reproduction in these short-day breeders is to provide a short-day signal that stimulates reproductive activity; this short-day signal can be easily given by means of a melatonin implant that causes the reinitiation of ovulation after 40 to 50 days (Kennaway, 1988; Chemineau et al., 1992). However, in some conditions, animals are refractory to a short-day signal and melatonin implant insertion has to be preceded by a long-day treatment to establish responsiveness to melatonin. One of the advantages of these photoperiodic treatments compared to the classically used hormonal treatments used to induce ovulation in females is that they can be used both for males and females and thus resulting fertility is improved (Chemineau et al., 1992). Melatonin has also been used in mink to advance the winter moult (Allain and Deletang, 1988). A new way may now be opened for a possible genetic selection of nonseasonal sheep by increasing the frequency of favorable alleles of the MT1 receptor gene (Pelletier et al., 2000).

CONCLUSION

By means of its circadian release of melatonin, the pineal gland is a key step in integrating the annual change in day length. Melatonin acts in discrete hypothalamic areas to control seasonal reproduction, but very little is known about the type of cells that melatonin targets. The cloning of different melatonin subtype receptors and the development of specific analogs should prove useful to identify the target cells of melatonin and the cellular mechanisms. Melatonin action on GnRH neurones is then mediated by a complex network of interneurones involving several neurotransmitter systems. Characterizing the interplay between these neural and endocrine pathways will give insights into the long-term mechanisms underlying the GnRH secretion control by melatonin.

REFERENCES

Allain D and Deletang F (1988) Interaction of age, strain and date of melatonin administration in the control of winter coat growth in mink. In *Proceedings of the 4th International Congress of Fur Animal Production*, pp 471-480, Rexdale, Canada.

Arendt J (1995) *Melatonin and the Mammalian Pineal Gland*, Chapman & Hall, London.

Bae HH, Mangels RA, Cho BS, Dark J, Yellon SM, and Zucker I (1999) Ventromedial hypothalamic mediation of photoperiodic gonadal responses in male syrian hamsters. J Biol Rhythms 14:391-401.

Baker JR and Ranson RM (1932) Factors affecting the breeding of the field mouse (*Microtus agrestis*): I. Light. Proc R Soc Lond B Biol Sci 110:313-322.

Barrell GK, Thrun LA, Brown ME, Viguié C, and Karsch FJ (2000) Importance of photoperiodic signal quality to entrainment of the circannual reproductive rhythm of the ewe. Biol Reprod 63:769-774.

Bartness TJ, Powers JB, Hastings MH, Bittman EL, and Goldman BD (1993) The timed infusion paradigm for melatonin delivery: What has it taught us about the melatonin signal, its reception, and the photoperiodic control of seasonal responses? J Pineal Res 15:161-190.

Bertrand F, Viguié C, Picard S, and Malpaux B (1998) Median eminence dopaminergic activation is critical for the early long-day inhibition of luteinizing hormone secretion in the ewe. Endocrinology 139:5094-5102.

Bittman EL (1993) The sites and consequences of melatonin binding in mammals. Am Zool 33:200-211.

Brydon L, Petit L, de Coppet P, Barrett P, Morgan PJ, Strosberg AD, and Jockers R (1999a) Polymorphism and signalling of melatonin receptors. Reprod Nutr Dev 39: 315-324.

Brydon L, Roka F, Petit L, de Coppet P, Tissot M, Barrett P, Morgan PJ, Nanoff C, Strosberg AD, and Jockers R (1999b) Dual signaling of human Mel1a melatonin receptors via G(i2), G(i3), and G(q/11) proteins. Mol Endocrinol 13:2025-2038.

Carter DS and Goldman BD (1983) Progonadal role of the pineal gland in the Djungurian hamster (*Phodopus sungorus sungorus*): Mediation by melatonin. Endocrinology 113:1268-1273.

Chabot V, Caldani M, de Reviers MM, and Pelletier J (1998) Localisation and quantification of melatonin receptors in the diencephalon and posterior telencephalon of the sheep brain. J Pineal Res 24:50-57.

Chemineau P, Malpaux B, Delgadillo JA, Guerin Y, Ravault JP, Thimonier J, and Pelletier J (1992) Control of sheep and goat reproduction: Use of light and melatonin. Anim Reprod Sci 30:157-184.

Concannon P, Roberts P, Baldwin B, and Tennant B (1997) Long-term entrainment of circannual reproductive and metabolic cycles by northern and southern hemisphere photoperiods in woodchucks (*Marmota monax*). Biol Reprod 57:1008-1015.

Coon SL, Zarazaga LA, Malpaux B, Ravault JP, Bodin L, Voisin P, Weller JL, Klein DC, and Chemineau P (1999) Genetic variability in plasma melatonin in sheep is due to pineal weight, not to variations in enzyme activities. Am J Physiol (Endocrinol Metab 40) 277:E792-E797.

Daveau A, Chemineau P, and Malpaux B (1999) Day-night rhythm in c-fos expression in the pre-mammillary hypothalamic area of the ewe. Soc Neurosci, October 23-28, Miami, Abstr 349-316.

Devries MJ, Ferreira SA, and Glass JD (1989) Evidence that short photoperiod-induced gonadal regression in the Mongolian gerbil is mediated by the action of melatonin in the medial hypothalamus. Brain Res 494:241-246.

Dowell SF and Lynch GR (1997) Duration of the melatonin pulse in the hypothalamus controls testicular function in pinealectomized mice (*Peromyscus leucopus*). Biol Reprod 36:1095-1101.

Dubocovich ML (1995) Melatonin receptors: Are there multiple subtypes? Trends Pharmacol Sci 16:50-56.

Ebling FJ, Alexander IH, Urbanski HF, and Hastings MH (1995) Effects of N-methyl-D-aspartate (NMDA) on seasonal cycles of reproduction, body weight and pelage colour in the male Siberian hamster. J Neuroendocrinol 7:555-566.

Glass JD and Lynch GR (1982) Evidence for a brain site of melatonin action in the white-footed mouse, *Peromyscus leucopus*. Neuroendocrinology 34:1-6.

Goldman BD (2001) Mammalian photoperiodic system: Formal properties and neuroendocrine mechanisms of photoperiodic time measurement. J Biol Rhythms 16:283-301.

Goldman BD and Nelson RJ (1993) Melatonin and seasonality in mammals. In *Melatonin: Biosynthesis, Physiological Effects, and Clinical Applications*, HS Yu and RJ Reiter, eds, pp 225-252, CRC Press, Boca Raton, FL.

Goodman RL (1994) Neuroendocrine control of the ovine estrous cycle. In *The Physiology of Reproduction*, E Knobil and JD Neill, eds, pp 659-709, Raven, New York.

Gorman MR and Zucker I (1998) Mammalian seasonal rhythms: New perspectives gained from the use of simulated natural photoperiods. In *Biological Clocks. Mecha-*

nisms and Applications, Y Touitou, ed, p 195, Elsevier Science, Amsterdam.

Granholm AC (1985) Effects of thyroid hormone deficiency on glial constituents in developing cerebellum of the rat. Exp Brain Res 59:451-456.

Hastings MH, Walker AP, Powers JB, Hutchison J, Steel EA, and Herbert J (1989) Differential effects of photoperiodic history on the responses of gonadotrophins and prolactin to intermediate day lengths in the male Syrian hamster. J Biol Rhythms 4:335-350.

Hastings MH, Walker AP, Roberts AC, and Herbert J (1988) Intra-hypothalamic melatonin blocks photoperiodic responsiveness in the male Syrian hamster. Neuroscience 24:987-991.

Hazlerigg DG, Morgan PJ, and Messager S (2001) Decoding photoperiodic time and melatonin in mammals: What can we learn from the pars tuberalis? J Biol Rhythms 16: 326-335.

Hewing M (1980) Cerebrospinal fluid-contacting area in the pineal recess of the vole (*Microtus agrestis*), guinea pig (*Cavia cobaya*), and Rhesus monkey (*Macaca mulatta*). Cell Tissue Res 209:473-484.

Hileman SM, Kuehl DE, and Jackson GL (1994) Effect of anterior hypothalamic area lesions on photoperiod-induced shifts in reproductive activity of the ewe. Endocrinology 135:1816-1823.

Howles CM, Craigon J, and Haynes NB (1982) Long-term rhythms of testicular volume and plasma prolactin concentrations in rams reared for 3 years in constant photoperiod. J Reprod Fertil 65:439-446.

Jansen HT and Jackson GL (1993) Circannual rhythms in the ewe: Patterns of ovarian cycles and prolactin secretion under two different constant photoperiods. Biol Reprod 49:627-634.

Kanematsu N, Mori Y, Hayashi S, and Hoshino K (1989) Presence of a distinct 24-hour melatonin rhythm in the ventricular cerebrospinal fluid of the goat. J Pineal Res 7:143-152.

Karsch FJ, Bittman EL, Foster DL, Goodman RL, Legan SJ, and Robinson JE (1984) Neuroendocrine basis of seasonal reproduction. Recent Prog Horm Res 40:185-232.

Karsch FJ, Dahl GE, Hachigian TM, and Thrun LA (1995) Involvement of thyroid hormones in seasonal reproduction. J Reprod Fertil Suppl 49:409-422.

Karsch FJ, Robinson JE, Woodfill CJI, and Brown MB (1989) Circannual cycles of luteinizing hormone and prolactin secretion in ewes during a prolonged exposure to a fixed photoperiod: Evidence for an endogenous reproductive rhythm. Biol Reprod 41:1034-1046.

Kennaway DJ (1988). Short- and long-term effects of manipulation of the pineal/melatonin axis in ewes. Reprod Nutr Dev 28:399-408.

Krajnak K and Nunez AA (1996) Short-photoperiod exposure reduces L-aromatic-amino-acid decarboxylase immunostaining in the arcuate nucleus and median eminence of male Syrian hamsters. Brain Res 712:95-101.

Kuljis RO and Advis JP (1989) Immunocytochemical and physiological evidence of a synapse between dopamine- and luteinizing hormone releasing hormone-containing neurons in the ewe median eminence. Endocrinology 124:1579-1581.

Le Corre S, Segu L, Caldani M, and Chemineau P (1994) Differences in ketanserin binding in the ventromedial hypothalamus of ewes responsive or refractory to short days. Neuroendocrinology 60:589-600.

Lee W, Watanabe M, and Glass JD (1995) Photoperiod affects the expression of neural cell adhesion molecule and polysialic acid in the hypothalamus of the Siberian hamster. Brain Res 690:64-72.

Lehman MN, Goodman RL, Karsch FJ, Jackson GL, Berriman SJ, and Jansen HT (1997) The GnRH system of seasonal breeders: Anatomy and plasticity. Brain Res Bull 44:445-457.

Lincoln GA and Clarke IJ (1994) Photoperiodically-induced cycles in the secretion of prolactin in hypothalamo-pituitary disconnected rams: Evidence for translation of the melatonin signal in the pituitary gland. J Neuroendocrinol 6:251-260.

Lincoln GA and Maeda KI (1992) Reproductive effects of placing micro-implants of melatonin in the mediobasal hypothalamus and preoptic area in rams. J Endocrinol 132:201-215.

Lincoln GA and Short RV (1980) Seasonal breeding: Nature's contraceptive. Recent Prog Horm Res 36:1-52.

Lincoln GA and Wu FCW (1991) Luteinizing hormone response to N-Methyl-D,L-Aspartate during a photoperiodically-induced reproductive cycle in the ram. J Repro Fertil 3:309-317.

Liu C, Weaver DR, Jin X, Shearman LP, Pieschl RL, Gribkoff VK, and Reppert SM (1997) Molecular dissection of two distinct actions of melatonin on the suprachiasmatic circadian clock. Neuron 19:91-102.

Malpaux B, Chemineau P, and Pelletier J (1993a) Melatonin and reproduction in sheep and goats. In *Melatonin: Biosynthesis, Physiological Effects, and Clinical Applications*, HS Yu and RJ Reiter, eds, pp 253-287, CRC Press, Boca Raton, FL.

Malpaux B, Daveau A, and Chemineau P (2000) Day-night changes in c-fos expression in the pre-mammillary hypothalamic area of the ewe are pineal dependent. Federation of European Neuroscience Societies, Abstr, June 24-28, Brighton, UK.

Malpaux B, Daveau A, Maurice F, Gayrard V, and Thiery JC (1993b) Short-day effects of melatonin on luteinizing hormone secretion in the ewe: Evidence for central sites of action in the mediobasal hypothalamus. Biol Reprod 48:752-760.

Malpaux B, Daveau A, Maurice-Mandon F, Duarte G, and Chemineau P (1998) Evidence that melatonin acts in the premammillary hypothalamic area to control reproduction in the ewe: Presence of binding sites and stimulation of luteinizing hormone secretion by in situ microimplant delivery. Endocrinology 139:1508-1516.

Malpaux B, Skinner DC, and Maurice F (1995) The ovine pars tuberalis does not appear to be targeted by melatonin to modulate luteinizing hormone secretion, but may be important for prolactin release. J Neuroendocrinol 7:199-206.

Malpaux B, Vigui C, Skinner DC, Thiéry JC, and Chemineau P (1997) Control of the circannual rhythm of reproduction by melatonin in the ewe. Brain Res Bull 44:431-438.

Maywood ES, Bittman EL, and Hastings MH (1996) Lesions of the melatonin- and androgen-responsive tissue of the dorsomedial nucleus of the hypothalamus block the gonadal response of male Syrian hamster to programmed infusions of melatonin. Biol Reprod 54:470-477.

Maywood ES and Hastings MH (1995) Lesions of the iodomelatonin-binding sites of the mediobasal hypothalamus spare the lactotropic, but block the gonadotropic response of male Syrian hamsters to short photoperiod and to melatonin. Endocrinology 136:144-153.

Nosjean O, Ferro M, Coge F, Beauverger P, Henlin JM, Lefoulon F, Fauchere JL, Delagrange P, Canet E, and Boutin JA (2000) Identification of the melatonin binding site MT3 as the quinone reductase 2. J Biol Chem 40:31311-31317.

Pelletier J, Auzan C, Daveau A, Clauser E, and Chemineau P (1999) Sheep 5HT2A receptors: Partial cloning of the coding sequence and mRNA localization by in situ hybridization in the ewe hypothalamus. Cell Tissue Res 295:231-239.

Pelletier J, Bodin L, Hanocq E, Malpaux B, Teyssier J, Thimonier J, and Chemineau P (2000) Association between expression of reproductive seasonality and alleles of the gene for Mel1a receptor in the ewe. Biol Reprod 62:1096-1101.

Pévet P (1988) The role of the pineal gland in the photoperiodic control of reproduction in different hamster species. Reprod Nutr Dev 28:443-458.

Reppert SM, Weaver DR, and Godson C (1996) Melatonin receptors step into the light: Cloning and classification of subtypes. Trends Pharmacol Sci 17:100-102.

Robinson JE and Karsch FJ (1988) Timing of the breeding season of the ewe: What is the role of day length? Reprod Nutr Dev 38:365-375.

Schwartz WJ, de la Iglesia H, Zlomanczuk P, and Illnerová H (2001) Encoding *Le Quattro Stagioni* within the mammalian brain: Photoperiodic orchestration through the suprachiasmatic nucleus. J Biol Rhythms 16:302-311.

Shaw PF, Kennaway DJ, and Seamark RF (1989) Evidence of high concentrations of melatonin in lateral ventricular cerebrospinal fluid of sheep. J Pineal Res 6:201-208.

Skinner DC and Malpaux B (1999) High melatonin concentrations in third ventricular cerebrospinal fluid are not due to galen vein blood recirculating through the choroid plexus. Endocrinology 140:4399-4405.

Steger RW, Juszczak M, Fadden C, and Bartke A (1995) Photoperiod effects on neurohypophyseal and tuberoinfundibular dopamine metabolism in the male hamster. Endocrinology 136:3000-3006.

Thiéry JC, Gayrard V, Le Corre S, Vigui C, Martin GB, Chemineau P, and Malpaux B (1995) Dopaminergic control of LH secretion by the A15 nucleus in anoestrous ewes. J Reprod Fertil Suppl 49:285-296.

Thiéry JC, Martin GB, Tillet Y, Caldani M, Quentin M, Jamain C, and Ravault JP (1989) Role of hypothalamic catecholamines in the regulation of luteinizing hormone and prolactin secretion in the ewe during seasonal anoestrus. Neuroendocrinology 49:80-87.

Tillet Y (1995) Distribution of neurotransmitters in the sheep brain. J Reprod Fertil Suppl 49:199-220.

Tricoire H, Locatelli A, Chemineau P, and Malpaux B (2000) Does melatonin reach the third ventricle directly from the pineal gland? Federation of European Neuroscience Societies, Abstr, June 24-28, Brighton, UK.

Urbanski HF and Pierce M (1991) Photoperiodic control on seasonal in Syrian hamsters: Involvement of excitatory amino acids receptors. Neuroendocrinol Lett 14:33-37.

Viguié C, Caraty A, Locatelli A, and Malpaux B (1995) Regulation of luteinizing hormone-releasing hormone (LHRH) secretion by melatonin in the ewe: II. Changes in N-Methyl-D,L-aspartic acid induced LHRH release during the stimulation of LH secretion by short days. Biol Reprod 52:1156-1161.

Viguié C, Jansen HT, Glass JD, Watanabe M, Billings HJ, Coolen L, Lehman MN, and Karsch FJ (2001) Potential for polysialylated form of neural cell adhesion molecule-mediated neuroplasticity within the gonadotropin-releasing hormone neurosecretory system of the ewe. Endocrinology 142:1317-1324.

Weaver DR, Liu C, and Reppert SM (1996) Nature's knockout: The Mel1b receptor is not necessary for reproductive and circadian responses to melatonin in Siberian hamsters. Mol Endocrinol 10:1478-1487.

Woodfill CJI, Wayne NL, Moenter SM, and Karsch FJ (1994) Photoperiodic synchronization of a circannual reproductive rhythm in sheep: Identification of season-specific time cues. Biol Reprod 50:965-976.

Zarazaga LA, Malpaux B, Bodin L, and Chemineau P (1998) The large variability in melatonin blood levels in ewes is under strong genetic influence. Am J Physiol (Endocrinol-Metabol 37) 274:E607-E610.

Zucker I (1985) Pineal gland influences period of circannual rhythms of ground squirrels. Am J Physiol 249:R111-R115.

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A 105- to 94-Kilodalton Protein in the Epididymal Fluids of Domestic Mammals Is Angiotensin I-Converting Enzyme (ACE); Evidence That Sperm Are the Source of This ACE[1]

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ABSTRACT

SDS-PAGE analysis of luminal fluid from the ram testis and epididymis revealed a protein of about 105 kDa in the fluid in the caput epididymal region. The molecular mass of this fluid protein shifted from 105 kDa to 94 kDa in the distal caput epididymidis and remained at 94 kDa in the lower regions of the epididymis. The possible sperm origin of this protein was suggested by the decrease in intensity of a 105-kDa compound on the sperm plasma membrane extract and by its total disappearance from the fluid of animals with impaired sperm production caused by scrotal heating.

The 94-kDa protein was purified from ram cauda epididymal fluid, and a rabbit polyclonal antiserum was obtained. This antiserum showed that membranes of testicular sperm and sperm from the initial caput were positive for the presence of an immunologically related antigen. The protein was immunolocalized mainly on the flagellar intermediate piece, whereas in some corpus and caudal sperm, only the apical ridge of the acrosomal vesicle was labeled.

The purified protein was microsequenced: its N-terminal was not found in the sequence database, but its tryptic fragments matched the sequence of the angiotensin I-converting enzyme (ACE). Indeed, the purified 94-kDa protein exhibited a carboxypeptidase activity inhibited by specific blockers of ACE. All the soluble seminal plasma ACE activity in the ram was attributable to the 94-kDa epididymal fluid ACE. The polyclonal antiserum also showed that a soluble form of ACE appeared specifically in the caput epididymal fluid of the boar, stallion, and bull. This soluble form was responsible for all the ACE activity observed in the fluid from the distal caput to the cauda epididymidis in these species.

Our results strongly suggest that the epididymal fluid ACE derives from the germinal form of ACE that is liberated from the testicular sperm in a specific epididymal area.

INTRODUCTION

When they leave the testis, mammalian spermatozoa are immobile and infertile. It is only after passing through the epididymis that sperm cells become motile and able to fuse with oocyte membranes [1, 2]. Acquisition of this fertilizing ability results from modifications of the sperm physiology in response to changes in the surrounding media due to the activity of the epididymal epithelium [1–3]. In the efferent ducts and caput epididymidis, almost all the proteins transported by the rete testis fluid are actively reabsorbed by the epithelium, as well as are water and some of the ions surrounding the sperm [1–5]. Thereafter, almost all the major changes in fluid proteins are due to epididymal secretions because of the existence of a hemato-epididymal barrier that precludes the exchange of proteins with blood and lymph [1–7].

During epididymal transit, an extensive remodeling of the sperm plasma membrane surface takes place in response to changes in the fluid. The sperm membrane is constituted of several well-defined domains established during spermatogenesis, and a redistribution of testicular sperm membrane proteins among these domains occurs during transit [1, 8]. Interactions between the sperm surface and the proteins of the luminal fluid also include integration of some of the secreted epididymal proteins [1, 2, 9, 10]. Finally, some of the surface compounds disappear, but the nature of these proteins and the mechanism of their removal remain to be elucidated [6, 9–11].

Using two-dimensional gel electrophoresis, we have recently compared the proteins of the luminal fluid of various regions of the ram and boar epididymis with ^{35}S-labeled proteins secreted by tissue from the same area in order to increase our understanding of the proteins that make up the epididymal fluid [3, 7, 12]. These studies have revealed that some compounds present in the fluid do not result from epididymal secretory activity. A protein of about 105 kDa appears in the fluid of the caput region in the boar and ram [6, 7]. Studies of variations in sperm surface proteins have also been performed in the ram [6, 9, 11]. These have shown that a compound of 115 kDa that is present on testicular sperm, and also on sperm from region 1 of the epididymis, is not found on the sperm membrane in the other regions [6, 11]. A similar sperm membrane compound has also been described in the boar [10]. These findings suggest a possible sperm origin of the 105-kDa fluid compound. We have therefore characterized this compound in order to ascertain its origin and obtain further information on its possible role in the sperm maturation process.

MATERIALS AND METHODS

Fluids and Sperm Collection

Epididymides and testes were surgically removed by castration or were obtained from freshly killed adult Ile de France rams, Large White and Meishan boars, Selle Français stallions, and Holstein bulls. Testicular and epididymal fluids were collected as previously described [13]. Spermatozoa were separated from the fluid (10 min, 10 000 × *g*), resuspended in PBS solution, and washed by two cycles of centrifugation (10 min, 500 × *g*). After dilution at about 10^8 spermatozoa/ml, sperm were deposed on a discontinuous PBS-Percoll gradient (40% and 90% layers) and centrifuged (10 min, 400 × *g*). Spermatozoa taken at the 90% interface were then washed twice, and the membrane was extracted by mixing the pellet with an equivalent volume of PBS containing 3% (w:v) *N*-octyl-β-D-glucopyranoside and the following protease inhibitors: 0.5 mM EDTA; 2

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mM para-aminobenzamidine; 50 μg/ml Nα-*p*-tosyl-L-lysine chloromethyl ketone; 10 μg/ml each of antipain, leupeptin, bestatin, E64, and pepstatin A. PMSF (0.5 mM) was added just before extraction. The mixture was left to stand at 4°C for 10–15 min; it was then centrifuged at 18 000 × *g* for 10 min. The supernatant was carefully removed and centrifuged again. The second supernatant was used directly or stored at −20°C. To measure the angiotensin I-converting enzyme (ACE) activity of the membrane extract, sperm were treated as described, but the extraction mixture was used without protease inhibitors. Activity was measured immediately after the second centrifugation.

Ram spermatogenesis was stopped by a 2°C elevation of the scrotum temperature using an insulated bag [14]. The bag was maintained in place continuously, and the animals were kept in a room with controlled light cycle and temperature (20–25°C). The decrease in sperm production was ascertained by the total number of sperm in ejaculates. After collection, seminal plasma was separated from sperm and/or cellular debris by centrifugation (two cycles at 18 000 × *g*, 10 min, 4°C), and the last supernatant was stored at −20°C until use.

Gel Electrophoresis and Protein Blotting

Sample preparation and methods for isoelectric focalization have previously been described [7]. Semi-dry transfer of proteins to nitrocellulose was performed over 2 h at 0.8 mA/cm^2. The blots were blocked with 20 mM Tris-HCl and 150 mM NaCl, pH 7.3 (TBS), supplemented with 0.5% Tween 20 and 5% goat serum. The second antibody was a goat anti-rabbit antibody conjugated with peroxidase (Institut Pasteur, Paris, France; dilution 1:2000 to 1:5000), and the peroxidase was revealed with 4-chloro-α-naphthol.

Purification of the 94-kDa Protein

The 94-kDa protein in the caudal fluid was purified by successive column chromatography using an HPLC system (Prosys; Biosepra, Villeneuve-la-Garenne, France). The first stage was a gel filtration column (Hiload 16/60, Superdex 200; Pharmacia, St. Quentin en Yveline, France), used with a flow of 0.7 ml/min and 0.5 M NaCl, 50 mM Tris-HCl (pH 7.5) buffered solution. The 94-kDa protein was obtained at a retention time corresponding to about 90–100 kDa, indicating that the protein was monomeric, as confirmed by gel electrophoresis under nonreducing conditions. The 94-kDa fractions were pooled and dialyzed against a mixture of Tris-HCl/Tris-base (50 mM) at pH 8.5 before concentration on a pressurized ultrafiltration device (Minicell; Amicon, Epernon, France) fitted with a 10-kDa cutoff membrane. The concentrated solution was loaded onto an ion-exchange chromatography column (Q-Hyper-D, 10 × 150 mm; Biosepra) equilibrated with the same buffer. Elution was started by a step at 10 mM NaCl (1 ml/min) for 5 min; then a 10–200 mM NaCl linear gradient was applied for 40 min (1 ml/min). The protein was desorbed at about 50–75 mM NaCl. The 94-kDa fractions were dialyzed against deionized water, vacuum dried, and dissolved in a mixture of 10% acetonitrile-0.1% trifluoroacetic acid (TFA) in deionized water. The reverse-phase column (C18, 5 μm, 300 A, 3.9 × 150 mm; Waters, St Quentin en Yveline, France) was subjected to a 10–100% linear gradient (0.5 ml/min) with buffer A (deionized water-0.1% TFA) and buffer B (90% acetonitrile-10% deionized water-0.1% TFA). One main peak was obtained at about 40–45% buffer B. The purity of the protein was assessed by one- and two-dimensional gel electrophoresis (not shown). The purified protein represented about 0.5% of the starting material (w:w).

N-Terminal Amino Acid Sequence Analysis

The N-terminal amino acid sequences of the protein and of its fragments separated by reverse-phase HPLC after tryptic digestion (trypsin sequencing grade from Sigma Chemical Co., St. Louis, MO; 1:100, w:w) were obtained on a Porton sequencer (Model LF3000; Beckman, Palo Alto, CA) using reagents and methods recommended by the manufacturer. Sequence homology research in data banks was performed using BLAST and FASTA software [15].

Antibody Production and Immunolocalization

Before the first injection, serum from different rabbits was tested on fluids from various epididymal regions by Western blotting. One presenting no reactions was chosen for immunization with the purified 94-kDa protein. Four injections were performed, each of about 100 μg of protein. The initial injection included Freund's complete adjuvant; 3 wk later, the first boost, including incomplete adjuvant, was given, followed by two more boosts at intervals of 15 days. The last serum was used for the experiments; it was diluted 1:10 000 with the ram fluids and 1:5000 with the ram membrane extracts and the boar, horse, and bull fluids.

Immunolocalization was performed on Percoll-washed spermatozoa from the same animal. The cells were incubated in TBS-10% goat serum and then with the anti-94-kDa polyclonal antibody (dilution 1:100). The incubated sperm were washed carefully by two centrifugations and resuspended in TBS-goat serum; they were then incubated for 30 min with a goat-anti-rabbit antibody labeled with fluorescein isothiocyanate (1:100 dilution; Institut Pasteur). Labeling was photographed under a fluorescence microscope using 400 ASA black and white film; all the fluorescent micrographs were photographed using the same exposure time. The preimmune serum and the second antibody alone were tested under the same conditions, and no reaction was observed with spermatozoa from the various zones.

ACE Activity Measurement

ACE activity was measured as described previously [16] using furanacryloyl-L-phenylalanylglycylglycine as substrate (FAPGG; Sigma). The activity of the 94-kDa protein was measured on the Q-hyperD fractions, which contained mainly the 94-kDa protein. Captopril and the ACE-inhibitory peptide P-Glu-Trp-Pro-Arg-Pro-Glu-Ile-Pro-Pro (both from Sigma) were dissolved in ethanol-dimethyl sulfoxide (50%-50%) at a concentration of 10^{-3} M.

RESULTS

Possible Sperm Origin of the 105- to 94-kDa Fluid Protein

Analysis by SDS-PAGE of perfused fluid from the ram testis and from various epididymal zones (Fig. 1) showed that a protein of about 105 kDa appeared in the fluid of the caput epididymal region (Fig. 2A); after increasing in intensity through several zones, this protein either changed in molecular mass or was replaced by a compound with a lower molecular mass of 94 kDa in the cauda epididymidis. Concomitantly, a compound of about 105 kDa decreased in

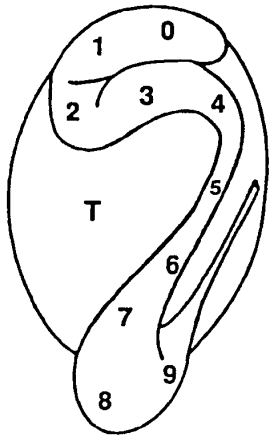

FIG. 1. Representation of the ram testis and epididymis. Fluids were collected from the testis (T) and by perfusion at various sites of the epididymis: caput (zones 0–3/4), corpus (zones 4/5–6), and cauda (zones 7–9) epididymidis.

intensity on the sperm plasma membrane as visualized on Coomassie blue-stained gel (Fig. 2B).

This protein was totally absent from two-dimensional gel electrophoresis of the epididymal fluid from azoospermic rams (Fig. 3) after a period of moderate heating of the scrotum [14]. The same result was also observed from a vas efferens-ligatured animal in which sperm transit was stopped (not shown).

These observations suggested a possible sperm origin for the 105- to 94-kDa epididymal fluid protein.

Immunocharacterization of the Protein

The 94-kDa protein was purified from ram cauda epididymal fluid proteins and used to obtain a monospecific rabbit polyclonal antibody (see *Materials and Methods*). The specificity of this anti-94-kDa protein antibody was tested on a nitrocellulose replica of a two-dimensional gel electrophoresis of the ram cauda epididymal fluid (Fig. 4). Only the spots forming the 94-kDa protein reacted with the antibody.

The polyclonal antibody did not react with any proteins in the rete testis fluid or the fluid of region 0 of the epididymis (Fig. 5A). The reaction with the protein was very faint in the epididymal fluid from region 1 but clearly visible in the fluids from zone 2 to the cauda epididymidis.

The antibody reacted with a protein of about 105 kDa in spermatozoa membrane extracts from the testis and all regions of the epididymis (Fig. 5B), showing that the two

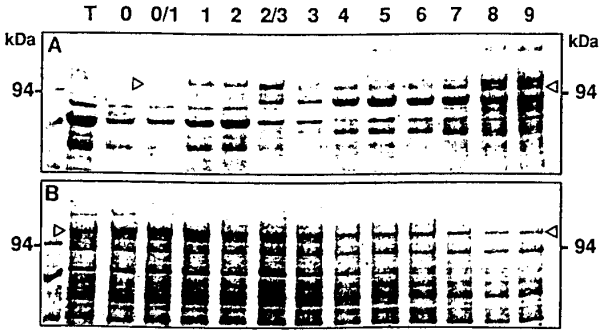

FIG. 2. Electrophoretic separation of epididymal ram fluid and sperm plasma membrane: 10 μg of epididymal fluid proteins (A) and of washed sperm plasma membrane extracts (B) from the testis (lane T) and the various epididymal zones (lanes 0–9) were separated on 6–16% SDS-PAGE, and gels were stained with Coomassie blue.

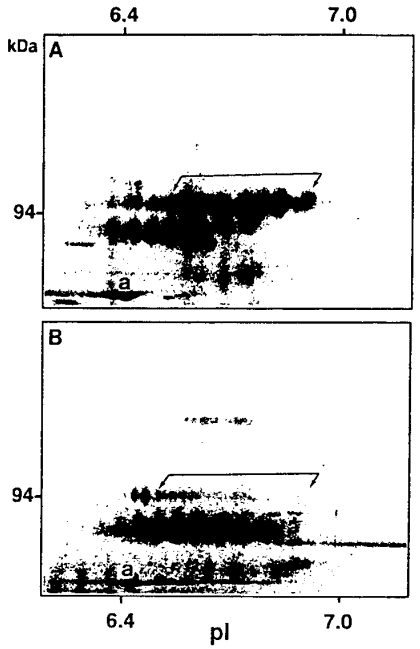

FIG. 3. Electrophoretic comparison of fluid from a normal ram and a ram with the scrotum heated. Regions of two-dimensional gel electrophoresis of fluids from zone 3 of the epididymis of a normal ram (A) and of a ram with the scrotum heated (B). Arrows indicate the position of the 105- to 94-kDa compound. a, Albumin. Silver-stained gels.

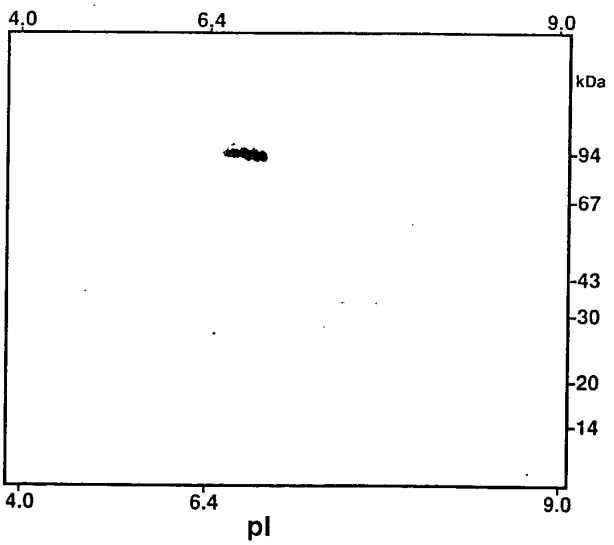

FIG. 4. Reactivity of the anti-94-kDa polyclonal antibody on two-dimensional blotting of ram cauda epididymal fluid. Fifty micrograms cauda epididymal fluid was separated after isoelectric focalization on a 6–16% SDS-PAGE gradient and transferred to nitrocellulose. The blot was probed with the anti-94-kDa polyclonal antibody.

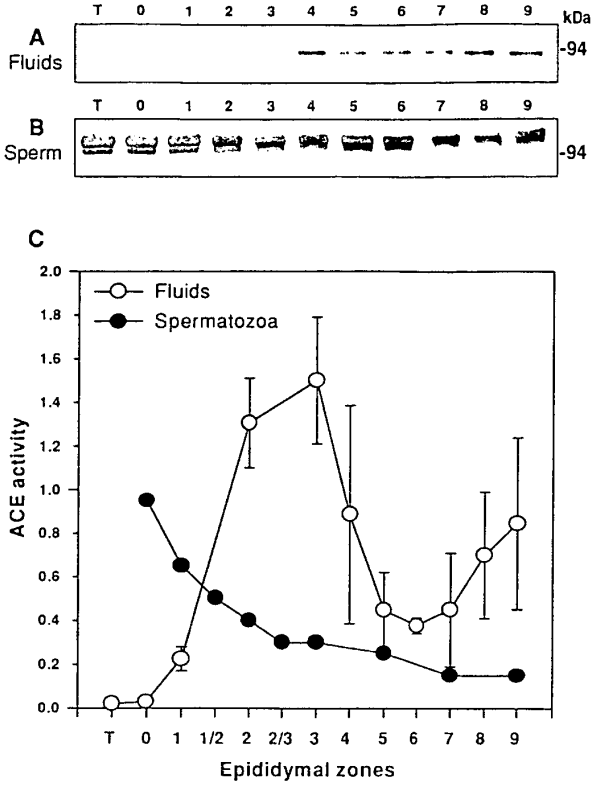

FIG. 5. Western blot reactivity with the anti-94-kDa polyclonal antibody and ACE activity of ram epididymal fluids and sperm membrane. A, B) Western blots of the fluids (A) and sperm membrane extracts (B) of ram rete testis (lane T) and the various epididymal zones (lanes 0–9) separated on 6–16% SDS-PAGE. Blots were incubated with the anti-94-kDa polyclonal antibody. Each lane contained 10 μg of protein. C) Measurements of the ACE activity in epididymal fluid samples (expressed in μmol of FAPGG/min per milligram) and sperm plasma membrane extracts (expressed in μmol of FAPGG/min per 10^7 spermatozoa). Fluid values are from two different animals; those for sperm are from one animal.

compounds were immunorelated. It is of note that there was a decrease in reaction intensity in membrane extracts from regions past the zone where the protein appeared in the fluid.

The antibody was also used to demonstrate that the apparent molecular mass of the protein changed from 105 kDa to 94 kDa during its passage from caput to corpus epididymidis (Fig. 6). The antibody reaction showed that the membrane and fluid proteins from zone 2 presented a slight difference in molecular mass and that the main shift in molecular mass to 94 kDa occurred between zone 2 and zone 4.

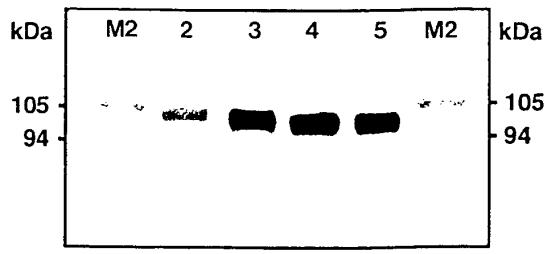

FIG. 6. Comparison of the molecular masses of ACE on sperm plasma membrane and in fluids during epididymal transit. Plasma membrane proteins extracted from sperm of zone 2 (lane M2) and fluids from zones 2, 3, 4, and 5 were run side by side on a 6–12% SDS-PAGE. The gel was blotted and revealed with the anti-94-kDa polyclonal antibody.

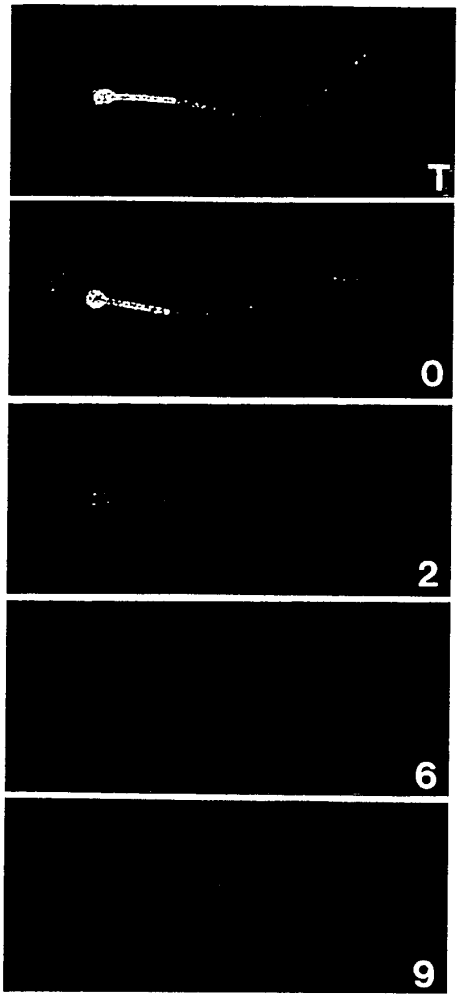

FIG. 7. Immunolocalization of ACE on testicular and epididymal sperm. Sperm from the testis (lane T) and the various epididymal zones (lanes 0, 2, 6, and 9) were incubated with the anti-94-kDa polyclonal antibody, and its localization was revealed with a second fluorescent antibody. Sperm were from the same animal. ×1000.

The anti-94-kDa polyclonal antibody produced intense immunolabeling on the intermediate piece of testicular sperm (Fig. 7, panel T) and on epididymal sperm from zone 0 (Fig. 7, panel 0) and zone 1 (not shown). A diffuse immunoreaction was also observed on the acrosomal cap. With sperm from zone 2 (Fig. 7, panel 2) and zone 3, the reaction on the intermediate piece was greatly decreased. After zone 3, only some sperm showed a positive reaction, which was restricted to the ridge of the acrosome (e.g., zone 6: Fig. 7, panel 6). This was unchanged in the subsequent zones (e.g., zone 9: Fig. 7, panel 9) and also in ejaculated sperm (not shown).

Biochemical Characterization

The ram cauda 94-kDa protein obtained from reverse-phase HPLC was subjected to N-terminal sequencing.

N-terminal of the 94 kDa protein
E-G-T-Q-G-Q-G-X-X-D-Q-G-X-X-D-Q-V-X-V-N-V-N-Q-A-

pep 42	-N-Y-Q-D-L-A-W-A-W-K-	100/100*
Bos	-T-S-R-N-Y-Q-D-L-A-W-A-W-K-S-W-R-D-	
	777 793	
pep 51-1	-A-L-H-H-E-Y-G-P-D-V-I-N-	83/100*
Bos	-V-R-R-A-L-H-R-H-Y-G-P-D-V-I-N-L-E-G-	
	857 874	
pep 36	-Q-G-W-T-P-K-	83/100*
Bos	-I-K-Q-G-W-T-P-L-R-M-	
	911 920	
pep 43	-E-G-A-N-P-G-F-H-E-A-I-G-D-V-K-	93/93*
Bos	-F-R-E-G-A-N-P-G-F-H-E-A-I-G-D-V-L-A-L-	
	1007 1025	
pep 51-2	-L-I-T-G-Q-S-V-M-S-A-A-Y	75/83*
Bos	-M-R-L-I-T-G-Q-S-N-M-S-A-S-A-M-M-	
	1185 1200	

FIG. 8. Comparison of the 94-kDa sequences with the angiotensin I-converting enzyme sequence from *Bos taurus*. *Identity/homology. The *Bos taurus* sequence is from [17]; Accession no. 1919242A in the Protein Research Foundation database.

Among the 24 successive amino acids obtained, 5 could not be identified (Fig. 8). This N-terminal sequence did not match any protein sequence in the databases. The protein was then submitted to tryptic cleavage, and four of the main peaks separated by reverse-phase chromatography were sequenced (Fig. 8): one of them showed 2 simultaneous sequences with an equivalent level of amino acids, indicating the presence of two fragments (peak 51). Sequences from these tryptic fragments were strongly homologous with the ACE sequence, and the best fit was found with the *Bos taurus* [17] protein (Fig. 8). This similarity also allowed matching of the amino acids of the two peptides of peak 51 with ACE sequences (Fig. 8).

The ACE activity of the ram cauda 94-kDa protein was tested using FAPPG as substrate [16]. Carboxypeptidase activity of about 20 μmol FAPGG converted per minute per milligram of protein was found within the 94-kDa peak fractions obtained by ion-exchange chromatography. This activity was inhibited by captopril (IC_{50} = 4.6 × 10^{-9} M) and by the peptide P-Glu-Trp-Pro-Arg-Pro-Glu-Ile-Pro-Pro (IC_{50} = 7 × 10^{-8} M), both of which are specific ACE inhibitors. These values were in good agreement with those previously published for ACE inhibition [18].

No ACE activity was found in the ram testicular fluid or the epididymal fluid from zone 0, where the 105- to 94-kDa protein was absent (Fig. 5C). Very low activity appeared between zones 0 and 1; a considerable increase occurred in the distal caput (zone 2 and 3), followed by a decrease in the corpus. The activity then slightly increased in the cauda fluid.

Because it was not possible to measure activity using intact sperm with the FAPGG method, the ACE activity was estimated on membrane extracts from sperm of the various epididymal zones. We observed a diminution during caput transit; then the activity remained almost constant until the end of the epididymis (Fig. 5C). Fluid and membrane extract activities were totally inhibited by captopril.

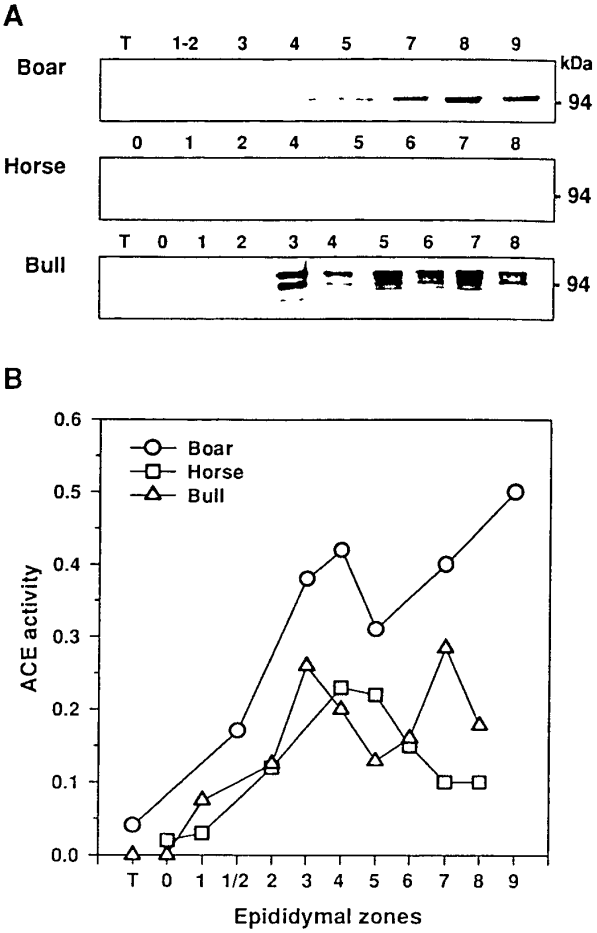

FIG. 9. Presence and activity of ACE in boar, horse, and bull epididymal fluids. A) Western blots of the boar, horse, and bull rete testis (lane T) and various epididymal fluids (lanes 0–9) separated on 6–16% SDS-PAGE. The blots were incubated with the anti-94-kDa polyclonal antibody. Each lane contained 10 μg of protein. B) Measurements of ACE activity in boar, horse, and bull epididymal fluid samples (expressed in μmol of FAPGG/min per milligram).

The Soluble Fluid Form of ACE Was Also Present in the Boar, Stallion, and Bull Epididymis

The presence of free ACE in the epididymal fluids of boars, stallions, and bulls was researched by immunoblotting with the ram anti-94-kDa protein antibody and by measuring the fluid carboxypeptidase activity. Immunoreactive bands at the expected molecular size were found in the fluids of these three species, with epididymal distribution similar to that observed in the ram (Fig. 9). In the boar, the protein was first weakly labeled on the blot at about 105 kDa in zone 1–2, and a shift in its molecular mass occurred in zone 3. A slight shift was also visible with the horse protein between zones 2 and 4. In the bull, two highly reactive bands were observed from the caput epididymidis: one at about 94 kDa and the other at a slightly higher molecular mass. Several other minor reactive bands were visible thereafter between the two main proteins.

The FAPPG-degrading activity of epididymal fluids from the boar, horse, and bull paralleled the presence of the 94-kDa protein in the fluid (Fig. 9). We also observed that the

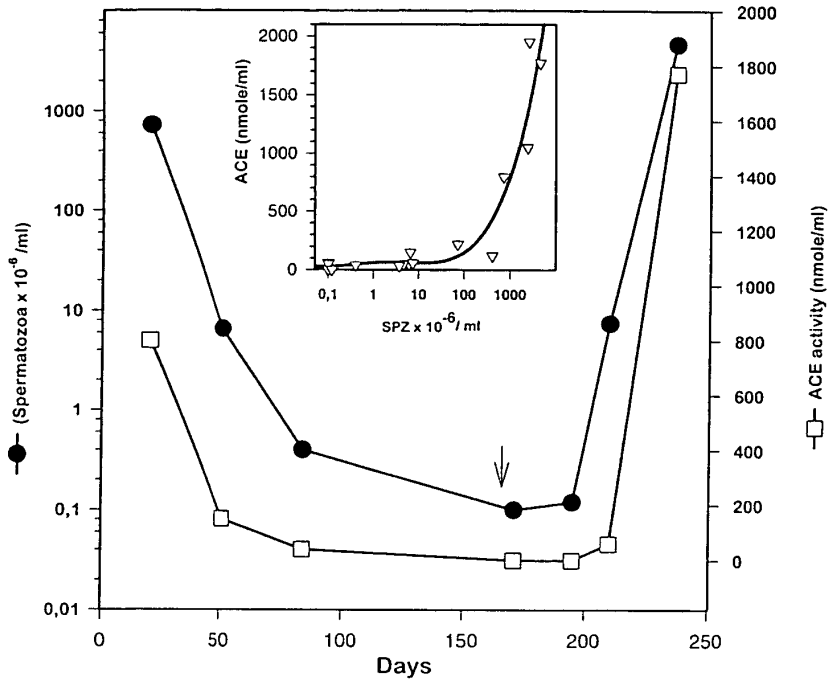

FIG. 10. ACE activity in ram seminal plasma. The seminal plasma of a ram with an insulated scrotum was obtained after ejaculation at various intervals during the sperm decrease phase as well as during the sperm recovery phase that occurred after removal of the bag (arrow). The number of sperm and the ACE activity were measured for each sample. The inset shows the relation between number of sperm and ACE activity in the seminal plasma for several heating and recovery phases.

ACE activity in the fluids was lower in these species than in the ram.

Relationship between Ram Seminal Plasma ACE and the Soluble Epididymal Form

The proportion of ACE activity due to the epididymal enzyme in ram seminal plasma was analyzed using a temporarily azoospermic animal. The variations in ACE activity always followed the number of sperm during the decreased sperm phase as well as during the increased sperm phase when scrotal heating was stopped. No activity could be measured when the sperm concentrations were lower than 10^6 sperm/ml (Fig. 10). The variations in activity in the seminal plasma were closely related to the presence of the 94-kDa protein. For example, when no enzyme activity was observed, no immunoreaction could be found in the seminal plasma on blotting even when a sensitive chemoluminescent method was used (not shown).

DISCUSSION

This study of ram epididymal fluid proteins showed that a protein of about 105 kDa appears specifically in the caput epididymidis. This protein remains in the fluid until the cauda epididymidis, and a change in its molecular mass occurs in the distal caput. This protein is also present in the seminal plasma. In order to characterize this protein and to elucidate its origin, we purified the 94-kDa compound from ram cauda epididymal fluid. Microsequencing demonstrated that this protein was the carboxypeptidase ACE (also known as kininase II and dipeptidyl peptidase A). Immunolocalization with a polyclonal antibody raised against the purified protein showed that an immunorelated compound was present on the testicular sperm membrane that disappeared when sperm passed through the caput epididymidis.

ACE is a ubiquitous membrane ectoprotein found in mammalian tissues [19, 20]. Two forms of this enzyme are known: one, of 150–180 kDa, is restricted to somatic tissues, including the epididymal epithelium and the prostate, while the second, of 90–110 kDa, is expressed exclusively in a stage-specific manner by haploid germ cells [19–21]. The somatic form is constituted of two homologous catalytic domains, named the N- and the C-terminal domains. The testicular form is restricted to the C-terminal domain, generated by the activity of a testis-specific promoter situated within the 12th intron of the somatic gene. This germinal form is characterized by a species-specific 65-amino acid N-terminal [19]. The C-terminal domains of both forms of the enzyme contain a hydrophobic amino acid sequence that allows cellular membrane insertion. It has been shown in somatic tissues (such as the lungs and kidneys) that the cell-bound ACE can become a free fluid enzyme after cleavage of this 7.5-kDa anchor [22].

Significant ACE activity has been reported in the genital tract of mammals, particularly in the epididymis [23–26]. Most of these studies have been based on enzyme activity measurements, and those concerning the epididymis are confusing, mainly because of the use of tissue extracts that included potential activity from the epithelium (and the surrounding tissues), blood and lymph, sperm cells, and epididymal fluid. Decrease in epididymal ACE activity was observed in rats after castration and ligature of efferent ducts, suggesting that the possible origin of ACE was germ cells; but the possible presence of a free form derived from the somatic ACE and transported by the testicular fluid could not be excluded in this experiment [27, 28].

Our findings and previous reports from the literature lead us to strongly suggest that the ACE present in the epididymal fluid is derived from the germinal cell membrane form and is released from the moment when sperm pass through the caput epididymidis. This membrane origin is supported

by our immunochemistry results showing the disappearance of the 105- to 94-kDa compound from the sperm membrane when the protein appears in the fluid. It was previously reported that an iodinated compound of about 115 kDa of ram testicular and epididymal zone 1 sperm membrane disappeared from the sperm in zone 2 [5, 11]. The 105- to 94-kDa fluid protein reported here could therefore represent this disappearing sperm membrane compound. We also observed that the 105- to 94-kDa protein was not secreted by the epithelium, since it was absent from autoradiography of ^{35}S-labeled proteins liberated into the fluid [12]. Moreover, because the 94-kDa protein had a specific N-terminal and a molecular mass within the range of the germinal ACE (110 kDa), this strongly suggests that it could not be derived from the somatic ACE present on epididymal epithelial cells (see also the previous paragraph). We also observed that the 105- to 94-kDa compound is recognized by an antibody directed against testicular sperm membrane with exactly the same epididymal distribution as obtained with the anti-94-kDa polyclonal antibody (unpublished results). Finally, each treatment that eliminated the sperm from the epididymal duct (such as scrotal heating, ligature of the vas efferens, or castration and testosterone supplementation [unpublished results]) resulted in the disappearance of this protein from the fluid.

When the protein is liberated in the epididymal fluid, there is a slight difference between its molecular mass and that of the sperm membrane protein; this suggests the loss of the plasma membrane anchor [22], which is in agreement with our assertion of a sperm origin (see Fig. 6). Thereafter, once the protein is in the fluid, the molecular mass further decreases, from about 105 to 94 kDa in the caput-corpus junction fluid. This shift could result from a change in the glycosylation state: the testicular enzyme is highly glycosylated [29], and our observations showed that the protein is polymorphic (see Figs. 3 and 4) and that it exhibits a change in molecular mass after glycosidase treatment (unpublished results). Partial proteolysis could also affect the molecular mass of the enzyme, but only further studies of the N- and C-terminal sequences from the 105- and 94-kDa forms will provide an answer. Both hypotheses remain therefore to be tested. It is of note that the molecular mass of the protein remaining on the sperm did not change throughout the epididymis.

In some cases, an immunoreactive compound migrating at less than 10 kDa from the main protein was observed in the fluid and on the membrane. The N-terminal of this protein was sequenced in the caudal fluid and was exactly the same as the N-terminal of the 94-kDa protein. This protein could represent a degradation product or a different post-transductional modification of the protein. These changes in the molecular mass of the protein or in the ratio between the quantity of this protein and the total proteins could be at the origin of the differences in ACE activity observed in the fluid throughout the epididymis.

The present results clearly establish that a soluble form of ACE exists in the epididymis of at least four different mammalian species (ram, boar, stallion, and bull), although slight differences in the molecular form and activity were seen. We have also observed an immunoreaction with a 94-kDa protein from the caudal fluid of rats with our anti-94-kDa polyclonal antibody (unpublished results). Furthermore, our study in the ram also showed that all the seminal plasma activity is apparently due to this epididymal free form.

Liberation of epididymal sperm ACE in the various domestic species studied occurred in a localized area, suggesting the need for a specific environment. ACE release in somatic tissue could be due to a cell plasma membrane-linked metalloprotease, named secretase (or sheddase), but this enzyme has yet to be characterized [22]. In the reproductive tract, proteolytic processing of several sperm membrane proteins during epididymal transit has already been described, and the role of the sperm intracellular proteases in this process has been suggested [30–34]. However, this concerns only posttesticular protein redistribution, where the degraded compounds remain inserted in the membrane and no massive release from the sperm surface has been described. Proteolytic enzymes are present in the testicular and epididymal fluids: for example, procathepsin L, a lysosomal precursor of a highly active protease, is secreted in the boar caput epididymidis [35] at the location where the ACE proteolytic process occurs. This enzyme has also been reported to be present in the acrosome of guinea pig spermatozoa [36]. However, in fluids in which proteolytic enzymes are detected, powerful inhibitors, such as α2-macroglobulin and cystatin C, are also found [37]. A disequilibrium between an enzyme and its inhibitors could be at the origin of in situ proteolytic activity.

The angiotensin-converting enzyme plays a critical role in blood pressure regulation via the degradation of the vasodilator bradykinin and the formation of angiotensin II. Angiotensin II can act on different types of receptors, and one of its main effects is the regulation of fluid and electrolyte homeostasis [28, 29]. Indeed, in vitro studies have shown that angiotensin II activates the ionic transport (particularly that of chloride ions) of cultured rat cauda epididymal cells [28, 29]. In the epididymis, angiotensin II and its receptors are immunolocalized mainly in the basal cells of the epididymal epithelium, with an increase from the caput to the cauda [28, 38]. In vivo, the main reabsorption of water and ions from the testis occurs in the efferent ducts [1, 2, 5] where no soluble ACE can be detected in the luminal fluid, but ACE activity on the sperm membrane might act in these regions to produce angiotensin II in the lumen. In vitro studies have also shown that the epithelium produces angiotensin I and transforms it into angiotensin II without the need for exogenous ACE, in agreement with the presence of somatic ACE on these cells [19, 21, 28]. Thus, in view of the regulation of ionic transport by the epithelium, the apparently large quantity of the enzyme liberated in the caput luminal fluid is certainly more than is needed for the formation of angiotensin II.

This is also sustained by the results of several groups that obtained ACE-deficient transgenic mice in which both somatic and germinal ACE were absent [39–41]. These animals had lowered blood pressure, and the males had impaired fertility although they had normal motile sperm. Such results indicate that sperm production is possible without ACE but that the sperm lack an unidentified differentiation step that prevents them from being fertile. The precise stage at which the absence of ACE interferes with fertility has not been determined, but it has been demonstrated that these sperm are unable to go through the female genital tract and then to bind to and fertilize the egg [40, 41]. A role for ACE has been suggested in the capacitation and acrosome reaction processes [26, 27, 42], and the angiotensin AT1 receptor was immunolocalized on the tail of rat and human sperm [43]. Meanwhile, it has recently been reported that angiotensinogen-deficient mice had normal fertility, although they did not produce angiotensin I, and hence angiotensin II—demonstrating that angiotensin II is

not necessary for the formation and activity of the epididymis and sperm in this species [41, 44]. All these results suggest that ACE is necessary for fertility but apparently not for its angiotensin II formation activity. Since ACE also has a number of other potential substrates (such as bradykinin and LHRH), it may have a carboxypeptidase activity toward other epididymal peptide(s) involved in sperm maturation.

Not all the testicular sperm ACE was released during transit; in our study ACE activity was found in membrane extracts of sperm from the corpus and the cauda epididymidis and also on ejaculated sperm. The remaining enzyme was immunolocalized on the acrosomal region of some of the ram sperm, and it has recently been shown in ejaculated horse and human sperm that the enzyme is situated within the acrosomal vesicle [45, 46]. This internal localization is supported by studies in which intact ejaculated boar and ram sperm activity in the degradation of bradykinin was very low [47]. This intraacrosomal location could also explain why the remaining sperm protein could not be iodinated after the caput epididymidis [6, 9]. Finally, the acrosomal location certainly protects the protein from proteolytic release in the epididymis and may explain its possible role in postejaculatory events [39–41].

In conclusion, we demonstrated biochemically that, like somatic membrane-bound ACE, germ cell membrane-bound ACE can be released in vivo in a soluble active form and that this release occurs in a very precise epididymal area. The important role played by this enzyme in reproductive function and in sperm physiology, and the mechanism of its epididymal release, remain to be clarified. This study also demonstrates that testicular sperm can be the vehicle for active compounds that can be released in a specific area of the reproductive tract.

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REFERENCES

1. Yanagimachi R. Mammalian fertilization. In: Knobil E, Neill JD (eds.), The Physiology of the Reproduction, 2nd edition. New York: Raven Press Ltd.; 1994: 189–317.
2. Turner TT. On the epididymis and its role in the development of the fertile ejaculate. J Androl 1995; 16:292–298.
3. Dacheux J-L, Druart X, Syntin P, Gatti J-L, Guerin Y, Dacheux F. Secretions and roles of epididymal proteins. Front Endocrinol 1995; 11:14–33.
4. Veeramachaneni DNR, Amann RP, Palmer JS, Hinton BT. Proteins in the luminal fluid of the ram excurrent ducts: changes in composition and evidence for differential endocytosis. J Androl 1990; 11:140–151.
5. Clulow J, Hansen LA, Jones RC. In vivo microperfusion of the ductuli efferentes testis of the rat: flow dependence of fluid reabsorption. Exp Physiol 1996; 81:633–644.
6. Dacheux J-L, Voglmayr JK. Sequence of sperm cell surface differentiation and its relationship to exogenous fluid proteins in the ram epididymis. Biol Reprod 1983; 29:1033–1046.
7. Syntin P, Dacheux F, Druart X, Gatti J-L, Okamura N, Dacheux J-L. Characterization and identification of proteins secreted in different regions of the adult boar epididymis. Biol Reprod 1996; 55:956–974.
8. Cowan AE, Nakhimovsky L, Myles DG, Koppel DE. Barriers to diffusion of plasma membrane proteins form early during guinea pig spermiogenesis. Biophys J 1997; 73:507–516.
9. Voglmayr JK, Sawyer RF, Dacheux J-L. Glycoproteins: a variable factor in surface transformations of ram spermatozoa during epididymal transit. Biol Reprod 1985; 33:165–176.
10. Dacheux J-L, Dacheux F, Paquignon M. Changes in sperm membrane and luminal proteins fluid during epididymal transit in the boar. Biol Reprod 1989; 40:635–651.
11. Volgmayr JK, Fairbanks G, Lewis RG. Surface glycoprotein changes in ram spermatozoa during epididymal maturation. Biol Reprod 1983; 29:767–775.
12. Druart X, Gatti J-L, Dacheux F, Dacheux J-L. Analysis by two-dimensional gel electrophoresis of ram epididymal secreted proteins. Cell Mol Biol 1994; 40:91–93.
13. Dacheux J-L. An in vitro perfusion technique to study epididymal secretion. IRCS Med Sci 1980; 8:137.
14. Mieusset R, Quintana Casares P, Sanchez Partida LG, Sowerbutts SF, Zupp JL, Setchell BP. Effects of heating the testis and epididymides of rams by scrotal insulation on fertility and embryonic mortality in ewes inseminated with frozen semen. J Reprod Fertil 1992; 94:337–343.
15. Altschul SH, Stephen F, Grish W, Miller W, Myers EW, Lipman DJ. Basic local alignment tool. J Mol Biol 1990; 215:403–410.
16. Holmquist B, Bünning P, Riordan JF. A continuous spectrophotometric assay for angiotensin converting enzyme. Anal Biochem 1979; 95: 540–548.
17. Shaw-Yung S, Fishel RS, Martin BM, Berk BC, Bernstein KE. Bovine angiotensin converting enzyme cDNA cloning and regulation. Increased expression during endothelial cell growth arrest. Circ Res 1992; 70:1274–1281.
18. Berecek KH, Zhang L. Biochemistry and cell biology of angiotensin-converting enzyme inhibitors. In: Mukhopadhyay AK, Raizada MK (eds.), Tissue Renin-Angiotensin Systems. New York: Plenum Press; 1995: 141–168.
19. Sibony M, Gasc J-M, Soubrier F, Alhenc-Gelas F, Corvol P. Gene expression and tissue localization of the two isoforms of angiotensin I-converting enzyme. Hypertension 1993; 21:827–835.
20. Costerousssse O, Jaspard E, Wei L, Corvol P, Alhenc-Gelas F. The angiotensin I-converting enzyme (kininase II): molecular organization and regulation of its expression in humans. J Cardiovasc Pharmacol 1992; 20(suppl 9):S10-S15.
21. Berg T, Sulner J, Lai CY, Soffer RL. Immunohistochemical localization of two angiotensin I-converting isoenzymes in the reproductive tract of the male rabbit. J Histochem Cytochem 1986; 34:753–760.
22. Hooper NM, Karran EH, Turner AJ. Membrane protein secretases. Biochem J 1997; 321:265–279.
23. Vanha-Perttula T, Mather JP, Bardin CW, Moss SB, Bellvé AR. Localization of the angiotensin-converting enzyme activity in testis and epididymis. Biol Reprod 1985; 33:870–877.
24. Hohlbrugger G, Schweisfurth H, Dahlheim H. Angiotensin I converting enzyme in rat testis, epididymis and vas deferens under different conditions. J Reprod Fertil 1982; 65:97–103.
25. Mukhopadhyay AK, Cobilanschi J, Brunswig-Spickenheier B, Leidenberger AF. Relevance of the tissue prorenin-renin-angiotensin system to male reproductive physiology. In: Mukhopadhyay AK, Raizada MK (eds.), Tissue Renin-Angiotensin Systems. New York: Plenum Press; 1995: 269–277.
26. Vinson GP, Saridogan E, Puddefoot JR, Djanhanbakhch O. Tissue renin-angiotensin systems and reproduction. Hum Reprod 1997; 12: 651–662.
27. Wong PYD, Uchendu CN. The role of angiotensin-converting enzyme in the rat epididymis. J Endocrinol 1990; 125:457–465.
28. Chan HC, Wong PYD. Paracrine-autocrine regulation of anion secretion in the epididymis: role of angiotensin II. Biol Signals 1996; 5: 309–316.
29. Ehlers MRW, Chen Y-N P, Riordan JF. The unique N-terminal sequence of testis angiotensin-converting enzyme is heavily O-glycosylated and unessential for activity or stability. Biochem Biophys Res Commun 1992; 183:199–205.
30. Petruszac JAM, Nehme CL, Bartles JR. Endoproteolytic cleavage in the extracellular domain of the integral plasma membrane protein CE9 precedes its redistribution from the posterior to the anterior tail of the rat spermatozoon during epididymal maturation. J Cell Biol 1991; 114:917–927.
31. Tulsiani DR, NagDas SK, Skudlarek MD, Orgebin-Crist M-C. Rat sperm plasma membrane mannosidase: localization and evidence for proteolytic processing during epididymal maturation. Dev Biol 1995; 167:584–595.
32. Jones R, Ma A, Hou S-T, Shalgi R, Hall L. Testicular biosynthesis and epididymal endoproteolytic processing of rat sperm surface. J Cell Sci 1996; 109:2561–2570.
33. Hunnicutt GR, Koppel DE, Myles DG. Analysis of the process of

localization of fertilin to the sperm posterior head plasma membrane domain during sperm maturation in the epididymis. Dev Biol 1997; 191:146–159.
34. Lum L, Blobel CP. Evidence for distinct serine protease activities with a potential role in processing the sperm protein fertilin. Dev Biol 1997; 191:131–145.
35. Okamura N, Tamba M, Uchima Y, Sugita Y, Dacheux F, Syntin P, Dacheux J-L. Direct evidence for the elevated synthesis and secretion of procathepsin L in the distal caput epididymis of boar. Biochim Biophys Acta 1995; 1245:221–226.
36. McDonald JK, Emerick JMC. Purification and characterization of procathepsin L a self-processing zymogen of guinea pig spermatozoa that act on a cathepsin D assay substrate. Arch Biochem Biophys 1995; 323:409–422.
37. Peloille S, Esnard A, Dacheux J-L, Guillou F, Gauthier F, Esnard F. Interactions between ovine cathepsin L, cystatin C and alpha 2-macroglobulin. Eur J Biochem 1997; 244:140–146.
38. Leung PS, Chan HC, Fu LXM, Zhou WL, Wong PYD. Angiotensin II receptors, AT1 and AT2 in the rat epididymis. Immunocytochemical and electrophysiological studies. Biochim Biophys Acta 1997; 1357: 65–72.
39. Esther CR, Marino EM, Bernstein KE. The role of angiotensin-converting enzyme in blood pressure control, renal function, and male fertility. Trends Endocrinol Metab 1997; 8:181–186.
40. Krege JH, John SWM, Langenbach LL, Hodgkin JB, Hagaman JR, Bachman ES, Jenette JC, O'Brien DA, Smithies O. Male-female differences in fertility and blood pressure in ACE-deficient mice. Nature 1995; 375:146–148.
41. Hagaman JR, Moyer JS, Bachman ES, Sibony M, Magyar PL, Welch JE, Smithies O, Krege JH, O'Brien DA. Angiotensin-converting enzyme and male fertility. Proc Natl Acad Sci USA 1998; 95:2552–2557.
42. Köhn F-M, Muller C, Drescher D, Neukamm C, El Mulla KF, Hagele W, Hinsch E, Habenicht UF, Schill W-B. Effect of angiotensin converting enzyme (ACE) and angiotensins on human sperm functions. Andrologia 1998; 30:207–215.
43. Vinson GP, Puddefoot JR, Ho MM, Barker S, Mehta J, Saridogan E, Djanhanbakhch O. Type 1 angiotensin receptors in rat and human sperm. J Endocrinol 1995; 144:369–378.
44. Tanimoto K, Sugiyama F, Goto Y, Ishida J, Takimoto E, Yagami K-I, Fukamizu A, Murakami K. Angiotensinogen-deficient mice with hypotension. J Biol Chem 1994; 269:31334–31337.
45. Dobrinsky I, Ignotz GG, Fagnan MS, Yudin AI, Ball BA. Isolation and characterization of a protein with homology to angiotensin converting enzyme from the periacrosomal plasma membrane of equine spermatozoa. Mol Reprod Dev 1997; 48:251–260.
46. Köhn F-M, Dammshäuser I, Neukamm C, Renneberg H, Siems W-E, Schill W-B, Aumüller G. Ultrastructural localization of angiotensin-converting enzyme in ejaculated human spermatozoa. Hum Reprod 1998; 13:604–610.
47. Heder G, Böttger A, Siems WE, Rottmann M, Kertscher U. The enzymatic degradation of bradykinin in semen of various species. Andrologia 1994; 26:295–309.