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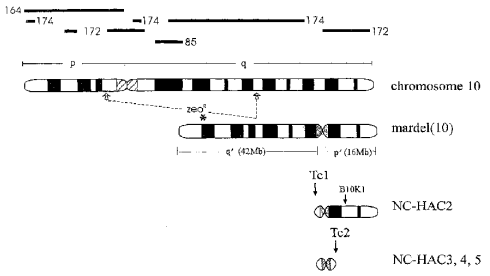
(54)【発明の名称】ネオセントロメアに基づくミニ染色体又は人工染色体

(57)【要約】
本発明は一般的にネオセントロメア又はその機能性誘導体又はその潜在型、合成型若しくはハイブリッド型を含む規定され若しくは単離された核酸分子に関するものであり、そしてとりわけ哺乳動物（例えば、ヒト）及び非哺乳動物のミニ染色体及び人工染色体を含むある範囲の真核生物のミニ染色体及び人工染色体を開発する場合におけるその使用に関する。本発明はミニ染色体を開発するためのテロメア関連染色体トランケーション（ＴＡＣＴ）アプローチを提供するが、これらのアプローチに限定されない。本発明はネオセントロメアを含むミニ染色体を作成する任意のトランケーションアプローチに関し、そして人工染色体の構築においてネオセントロメア機能を提供するクローニングされ又は予め作成されたＤＮＡを用いる任意のトランスフェクションアプローチにも関する。このようなミニ染色体及び人工染色体はある範囲の遺伝子治療に有用である。



【特許請求の範囲】
【請求項１】
真核生物染色体に由来するヌクレオチド配列を含み且つネオセントロメアを含む単離された核酸分子であって、適合する細胞中でトランケーション型若しくは改変型として存在し、又は適合細胞中へ予め作成されたＤＮＡ実体としてトランスフェクションにより導入された場合、複製でき、染色体外因子として作用でき且つ細胞分裂と共に分離できる核酸分子。
【請求項２】
真核生物染色体がヒト若しくは霊長類などの哺乳動物又は植物、鳥類、昆虫、ぜん虫、真菌、酵母又は爬虫類に由来するものである、請求項１記載の単離された核酸分子。
【請求項３】
真核生物染色体がヒトに由来するものである、請求項２記載の単離された核酸分子。
【請求項４】
真核生物染色体が家畜動物に由来するものである、請求項２記載の単離された核酸分子。
【請求項５】
該分子がマーデル（１０）染色体又はその等価物のｑ’腕の標的化されたテロメア関連トランケーション及びｐ’腕の無作為トランケーションの結合によって作成されるものである、請求項１記載の単離された核酸分子。
【請求項６】
該核酸分子が染色体１０又はその等価物の上のｑ２４とｑ２６の間に等しい位置にネオセントロメアを含むものである、請求項５記載の単離された核酸分子。
【請求項７】
該核酸分子が染色体１０又はその等価物の上のｑ２５に等しい位置に又はほぼｑ２５に等しい位置にネオセントロメアを含むものである、請求項６記載の単離された核酸分子。
【請求項８】
該核酸分子が染色体１０又はその等価物の上のｑ２５．２に等しい位置に又はほぼｑ２５．２に等しい位置にネオセントロメアを含むものである、請求項７記載の単離された核酸分子。
【請求項９】
ネオセントロメアに対応する領域がα－サテライトＤＮＡを実質的に欠いているものである、請求項１又は請求項６又は請求項７又は請求項８記載の核酸分子。
【請求項１０】
核酸分子が約０．５から約２．０Ｍｂのサイズである、請求項９記載の核酸分子。
【請求項１１】
核酸分子が約０．８から約１．６Ｍｂのサイズである、請求項１０記載の核酸分子。
【請求項１２】
ネオセントロメア又は機能性の相同染色体を同定する方法であって、哺乳動物のＣＥＮＰ－Ａ及び／又はＣＥＮＰ－Ｃに特異的な抗体又は哺乳動物のＣＥＮＰ－Ａ及び／又はＣＥＮＰ－Ｃと交差相互作用できる抗体を用いるクロマチン免疫沈降によりＤＮＡを単離する工程、免疫沈降により単離されたＤＮＡを増幅する工程且つ増幅されたＤＮＡの中に標識を組み込む工程、次いで増幅されたＤＮＡを用いてゲノムＤＮＡ又はその等価物を含むＤＮＡアレーを精査する工程、及び該免疫沈降したＤＮＡにハイブリダイズするクローンを同定し単離する工程を含む方法。
【請求項１３】
哺乳動物がヒト、家畜動物、愛玩動物又は実験室試験動物である、請求項１２記載の方法。
【請求項１４】
哺乳動物がヒトである、請求項１３記載の方法。
【請求項１５】
ネオセントロメアが染色体１０又はその等価物の上のｐ２４とｐ２６の間に等しい位置に

あるものである、請求項１４記載の方法。
【請求項１６】
ネオセントロメアが染色体１０又はその等価物の上のｑ２５又はほぼｑ２５の位置にあるものである、請求項１５記載の方法。
【請求項１７】
ネオセントロメアが染色体１０又はその等価物の上のｑ２５．２又はほぼｑ２５．２の位置にあるものである、請求項１６記載の方法。
【請求項１８】
ヒトのネオセントロメアに基づくミニ染色体（ＮＣ－ＭｉＣｓ）の形態の単離された核酸分子であって、該ＮＣ－ＭｉＣｓが細胞分裂の間に安定に分離できるネオセントロメア又はその潜在型、合成型若しくはハイブリッド型を含むものである核酸分子。
【請求項１９】
マーデル（１０）染色体又はその等価物のｑ’腕の標的化されたテロメア関連トランケーション及びｐ’腕の無作為トランケーションの結合によって作成される、請求項１８記載の単離された核酸分子。
【請求項２０】
ネオセントロメアが染色体１０又はその等価物の上のｑ２４とｑ２６の間に等しい位置にあるものである、請求項１９記載の単離された核酸分子。
【請求項２１】
ネオセントロメアが染色体１０又はその等価物の上のｑ２５又はほぼｑ２５の位置にあるものである、請求項２０記載の単離された核酸分子。
【請求項２２】
ネオセントロメアが染色体１０又はその等価物の上のｑ２５．２又はほぼｑ２５．２の位置にあるものである、請求項２０記載の単離された核酸分子。
【請求項２３】
ミニ染色体を作成する方法であって、
ネオセントロメアを含む染色体を保持するヒト若しくは哺乳動物の細胞の中に、テロメア配列、選択可能マーカー、標的染色体のｑ’腕又はｐ’腕の一方又は他方の上の相同なターゲティングＤＮＡを有するベクターを含むトランケーション構築物を導入する工程、
選択可能マーカーを発現する細胞を選択する工程、
該細胞に、該ｑ’又はｐ’ターゲティングＤＮＡの他方を含む第２のトランケーション構築物を導入する工程、
該第２のトランケーション構築物と関連する選択可能マーカーを発現する細胞を選択する工程、
次いで、ネオセントロメアを含むトランケーションされた染色体を単離する工程、
を含む方法。
【請求項２４】
細胞がヒトの細胞である、請求項２３記載の方法。
【請求項２５】
ネオセントロメアが染色体１０又はその等価物の上のｑ２４とｑ２６の間に等しい位置にあるものである、請求項２４記載の方法。
【請求項２６】
ネオセントロメアが染色体１０又はその等価物の上のｑ２５又はほぼｑ２５の位置にあるものである、請求項２４記載の方法。
【請求項２７】
ネオセントロメアが染色体１０又はその等価物の上のｑ２５．２又はほぼｑ２５．２の位置にあるものである、請求項２４記載の方法。
【請求項２８】
ネオセントロメアを挟んでいるｑ’及びｐ’腕を有する染色体を含む単離された細胞であって、ｑ’及び／又はｐ’腕の一方又は両方がトランケーションされているものである細

胞。
【請求項２９】
受託番号００１２２００１（ＣＨＯ／ＢＥＺＢ３０）としてＥＣＡＡＣに寄託された単離された細胞系統。
【請求項３０】
受託番号００１２２００２（ＨＴ１０８０－ＭＩＣ１）としてＥＣＡＡＣに寄託された単離された細胞系統。
【請求項３１】
受託番号００１２２００３（ＨＴ１０８０－ＭＩＣ２）としてＥＣＡＡＣに寄託された単離された細胞系統。
【請求項３２】
受託番号００１２２００４（ＨＴ１０８０－ＭＩＣ３）としてＥＣＡＡＣに寄託された単離された細胞系統。
【請求項３３】
受託番号００１２２００５（ＨＴ１０８０－ＭＩＣ４）としてＥＣＡＡＣに寄託された単離された細胞系統。
【請求項３４】
受託番号００１２２００６（ＨＴ１０８０－ＭＩＣ５）としてＥＣＡＡＣに寄託された単離された細胞系統。
【請求項３５】
受託番号００１２２００７（ＨＴ１０８０－ＭＩＣ５ａ）としてＥＣＡＡＣに寄託された単離された細胞系統。
【請求項３６】
受託番号００１２２００８（ＨＴ１０８０－ＭＩＣ５ｂ）としてＥＣＡＡＣに寄託された単離された細胞系統。
【請求項３７】
遺伝子治療に使用するためのミニ染色体の製造における請求項１記載の単離された核酸分子の使用。
【請求項３８】
ミニ染色体がＮＣ－ＭｉＣである請求項３７記載の使用。
【請求項３９】
ミニ染色体がＹＡＣである請求項３７記載の使用。
【請求項４０】
ミニ染色体がＨＡＣである請求項３７記載の使用。
【請求項４１】
ミニ染色体がＭＡＣである請求項３７記載の使用。
【請求項４２】
ミニ染色体がＰＬＡＣである請求項３７記載の使用。
【発明の詳細な説明】
【技術分野】
【０００１】
本発明は一般的にネオセントロメア又はその機能的誘導体又はその潜在型、合成型若しくはハイブリッド型を包含する規定された若しくは単離された核酸分子、及びとりわけ哺乳動物（例えば、ヒト）及び非哺乳動物のミニ染色体及び人工染色体を含むある範囲の真核生物のミニ染色体及び人工染色体を開発する場合におけるその使用に関する。本発明はさらに、ミニ染色体を開発するためのテロメア関連染色体トランケーション（ＴＡＣＴ）アプローチを提供するが、このアプローチに限定されるわけではない。本発明は、ミニ染色体を含むネオセントロメアを調製する如何なるトランケーションアプローチにも関連し、あるいは人工染色体の構築においてネオセントロメア機能を提供するクローニングされ若しくは予め構築されたＤＮＡを用いる如何なるトランスフェクションアプローチにも関連

する。このようなミニ染色体及び人工染色体は遺伝子治療の分野でも有用である。
【背景技術】
【０００２】
本明細書中で数値で引用した出版物の書誌的事項の詳細は明細書の末尾に集めてある。
【０００３】
本明細書における如何なる先行技術への参照も、その先行技術がオーストラリアにおいて又は如何なる他の国においても通常の一般的知識の一部を構成するとの認識又は示唆を示すものではなく、そしてそう解すべきでもない。
【０００４】
組換えＤＮＡ技術の急速に発展する精緻化は研究や医学分野・関連厚生分野における進歩を著しく促進した。とりわけ重要な領域はヒト遺伝学を含む哺乳動物遺伝学においてであり、遺伝的異常の背後に潜む分子的機序の解明においてである。この分野での研究の進歩はヒトのセントロメアを含む核酸分子の完全な配列決定が欠如していることにより妨げられてきた。このような分子の同定及び／又はクローニングはヒト細胞を含む真核細胞や特定の哺乳動物中に遺伝子を導入するための技術の発達を促進するであろうし、そして遺伝子治療や哺乳動物細胞においてある範囲の組換え遺伝子産物を生産するための発現系の開発にとっての重要な財産となるであろう。完全に配列決定されたセントロメアの同定及び／又はクローニングは哺乳動物のミニ染色体及び人工染色体の開発を促進することが重要である。
【０００５】
哺乳動物のミニ染色体及び人工染色体は、エピソーム的に存在しそして宿主のゲノムＤＮＡから独立にそれらの内因性制御因子の下で遺伝子を発現させうるそれらの能力に基づいて、多様な潜在的バイオテクノロジー的応用及び治療分野での応用を有する。これらは実質的に完全に機能的な哺乳動物染色体であるから、これらの実体中に導入され得るＤＮＡのサイズには理論的上限は存在しない。これらの酵母の同種体（人工染色体）との類比から、哺乳動物のミニ染色体及び人工染色体は、正常な分離のためには、機能的な哺乳動物セントロメア、テロメア及びＤＮＡ複製起点を必要とすると仮定されてきた。現時点で、これらの三つの成分の中で最も理解されておらず且つ最も複雑なものはセントロメアである。
【０００６】
正しいセントロメア活性に必要なタンパク質成分の同定（その数は増加しつつある）及び多様な種におけるセントロメアＤＮＡ配列の特性決定はセントロメアの形成及び機能の根底にある機序についての知識を顕著に増加させた（非特許文献１，２，３参照）。この知識は哺乳動物のミニ染色体及び人工染色体の構築のための幾つかの戦略の開発を促進させた。一つの戦略は、ヒトα－サテライトの大きなアレーを持つテロメアＤＮＡをヒト細胞中に共トランスフェクションさせることによるヒト人工染色体の新たな形成を含む（非特許文献４，５，６，７）。この戦略を用いる研究により、ＣＥＮＰ－Ｂボックスを含むα－サテライトＤＮＡのみが新たな人工染色体形成に関与できることが明らかにされた（非特許文献５，８）。一般化された人工染色体のうちの一部はその構造が線形であるが（４）、他のものは常に環状であった（非特許文献５，７，８）。これらの人工染色体はそのサイズが約１Ｍｂ～１３Ｍｂの範囲であり、通常注入したＤＮＡよりも１オーダー以上大きかった。このサイズの増加はトランスフェクション後の注入ＤＮＡの末端結合によるものであった（非特許文献４）。
【０００７】
別の１戦略は、正常なセントロメアの周りの非本質的な染色体様物質を除去してミニ染色体をインサイチュ生産するために、テロメア関連染色体トランケーションの使用を含む。ヒトＸ染色体の配列トランケーションは、Ｘ染色体αサテライトＤＮＡの約１．８Ｍｂ及び近位のＸｐＤＮＡの４００ｋｂを含む２．５Ｍｂのミニ染色体を生じた（非特許文献９，１０，１１）。この染色体は正常なヒトＸ染色体のそれに匹敵する有糸分裂安定性を示す（非特許文献１０，１１）。同様のアプローチにより、ヒトＹ染色体に由来したミニ染

色体が幾つか作られた。それらのサイズは約０．７Ｍｂから４Ｍｂを超える程の範囲であり、サイズのより小さなものは比較的不安定であった（非特許文献１２，１３）。より大きなミニ染色体はＣＨＯ細胞、ニワトリＤＴ４０細胞及びマウスＬ細胞中で安定に維持されたが、マウスＥＳ細胞中に導入されると安定性が悪くなった、これは細胞型が異なると正しいセントロメア機能に対する要求も異なることを示唆する（非特許文献１４，１５）。

【０００８】

哺乳動物の人工染色体を調製するための第３の戦略は、近点のＤＮＡの増幅及びそれに続く染色体の制御された切断により６０と４００Ｍｂの間のサテライトＤＮＡに基づく人工染色体を調製する工程を含む（非特許文献１６，１７，１８，１９，２０）。

【０００９】

近年、セントロメア機能と通常関連付けられてきた反復配列を欠如するネオセントロメア類（ＮＣｓ）がヒト（非特許文献２１）及びドロソフィラ（非特許文献２２）中で記述された。基礎をなすＤＮＡの特性決定及びヒトにおけるネオセントロメアのタンパク質結合の様相はＤＮＡ一次配列組成とは無関係なセントロメア形成の後生的な機序を示唆した（非特許文献２３，２４，２５，２６）。ネオセントロメアの発見は、セントロメアＤＮＡの反復配列の使用に基づいて従来記述されたきたアプローチに替わる、ミニ染色体及び人工染色体の構築のためのアプローチを提供する。

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【非特許文献１７】
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【発明の開示】
【発明が解決しようとする課題】
【００１０】
本発明者らは完全に機能的なヒトネオセントロメアを含む有糸分裂に対し安定な一連のヒトミニ染色体を作成することを目的とする。ヒトミニ染色体の作成は遺伝子治療、トランスジェニック植物及び動物の生産、及び組換えタンパク質の生産の発展を可能とする。
【課題を解決するための手段】
【００１１】
発明の概要
本明細書を通じて、論旨が別意を要求しない限り、語「含む（comprise）」又は「含む(comprises)」若しくは「含む(comprising)」などのその変形は述べられた要素若しくは整数又は要素若しくは整数の群の含有を意味するが他の任意の要素若しくは整数又は要素若しくは整数の群の排除を意味するものではないと理解される。
【００１２】
ヌクレオチド配列及びアミノ酸配列は配列番号の数（配列番号：）により引用される。この配列番号：は配列同定子＜４００＞１、＜４００＞２などに数値的に対応している。配列表は特許請求の範囲の後に添付される。
【００１３】
本発明はテロメア関連染色体トランケーション（ＴＡＣＴ）アプローチを使用してミニ染色体を開発することに部分的に基づいている。選択可能マーカー、ヒトのマーデル（mardel）（１０）などの真核生物染色体のｐ'又はｑ'腕の上の小さな領域に相同なターゲティングＤＮＡ配列、及びテロメア配列の小さなアレーを含むトランケーション構築物が開発される。ｐ'及びｑ'腕はこのマーデル（１０）染色体の短い腕及び長い腕を指すが、一般的にはネオセントロメアを含む任意のマーカー染色体の短い腕及び長い腕をも指す。第１のトランケーション構築物を標的細胞中にトランスフェクトし、そのマーカーを選択する。これは、使用したトランケーション構築物に応じて先端切断されたｐ'及びｑ'腕を持つ標的染色体を結果として生ずる。次に他のｐ'及びｑ'腕のトランケーション構築物を用いて同じ細胞の中に第２のトランケーション構築物をトランスフェクトする。再び、選択した後、該標的染色体のｐ'及びｑ'腕のトランケーションされた形が得られる。

次いで、得られたミニ染色体を単離し、遺伝子治療又は遺伝子発現のために使用しうる。テロメアＤＮＡを含む若しくは含まない修正標的化法を用いて遺伝子若しくは他のヌクレオチド配列を標的染色体中に導入してもよい。

【００１４】

本発明の一つの側面は真核生物のネオセントロメアを規定するヌクレオチド配列を含む単離された核酸分子を提供する。

【００１５】

より具体的には、本発明は真核生物染色体に由来するヌクレオチド配列を含み且つネオセントロメアを含む単離された核酸分子であって、適合細胞中でトランケーション型若しくは改変型で存在し、又は適合細胞中に予め作成されたＤＮＡ実体としてトランスフェクションにより導入された場合複製でき、染色体外因子として作用でき且つ細胞分裂と共に分離することができる核酸分子を提供する。

【００１６】

本発明の別の一側面はネオセントロメア又は機能的同族体を同定する方法であって、哺乳動物ＣＥＮＰ－Ａ及び／又はＣＥＮＰ－Ｃに特異的な抗体又は哺乳動物ＣＥＮＰ－Ａ及び／又はＣＥＮＰ－Ｃと交差相互作用できる抗体を用いるクロマチン免疫沈降法によりＤＮＡを単離する工程、免疫沈降法により単離されたＤＮＡを増幅する工程、及び増幅されたＤＮＡ中に標識を組み込む工程、次いで増幅されたＤＮＡを用いてゲノムＤＮＡ若しくはその等価物を含むＤＮＡアレー精査する工程、及び該免疫沈降したＤＮＡにハイブリダイズするクローンを同定し且つ単離する工程を含む方法の使用を意図する。

【００１７】

本発明のさらなる側面は、細胞分裂の間に安定に分離できるネオセントロメア又はその潜在型、合成型若しくはハイブリッド型を含むヒトネオセントロメアに基づくミニ染色体（ＮＣ－ＭｉＣｓ）の形態で単離された核酸分子を提供する。

【００１８】

本発明のさらに別の一側面は、ミニ染色体を作成する方法であって、

ネオセントロメアを含む染色体を保持するヒト又は哺乳動物の細胞中に、標的染色体のｑ'及びｐ'腕の一方又は他方の上に、テロメア配列、選択可能マーカー、ターゲティング相同ＤＮＡを有するベクターを含むトランケーション構築物を導入する工程、

選択可能マーカーを発現する細胞を選択する工程、

該細胞の中に該ｑ'又はｐ'のターゲティングＤＮＡの他方を含む第２のトランケーション構築物を導入する工程、

該第２のトランケーション構築物と関連する選択可能マーカーを発現する細胞を選択する工程、及び

次いで、ネオセントロメアを含むトランケーションされた染色体を単離する工程、

を含む方法を提供する。

【発明を実施するための最良の形態】

【００１９】

本発明はネオセントロメア特性を示す核酸分子の同定及び単離に一部基づいている。本発明によれば、ネオセントロメアは正常なセントロメア（例えば、ヒトにおけるα－サテライトやマウスにおけるマイナーサテライト）の上に存在する反復ＤＮＡ配列を実質的な量として含まず、活性化されるとセントロメアとして機能することができるセントロメアであると考えられる。例えば、哺乳動物（例えば、ヒト）のネオセントロメアはα－サテライトＤＮＡ反復配列を実質的に含まないセントロメアである。用語「実質的な」とは、この論旨において、該核酸分子が培地のストリンジェンシー条件の下でのＦＩＳＨ分析により又は培地の相同性基準の下での直接配列比較により、α－サテライトなどの正常なセントロメア反復ＤＮＡ配列を検出可能な程に含まないことを意味する。しかしながら、このネオセントロメアは高度に分岐した（highly diverged)正常セントロメア反復ＤＮＡ配列を少数含みうる。例えば、霊長類においては、α－サテライトＤＮＡは長さが約１７０ｂｐであると考えられる。活性化されたネオセントロメア又は他の方法でセントロメアとし

て機能するネオセントロメアを含む核酸分子は、本発明に従ってミニ染色体又は他のＤＮＡと共に予め作成された形態の核酸分子がトランスフェクションし、複製し、染色体外に留まり、細胞分裂と共に分離することを容易にする。本明細書で「ネオセントロメア」と言うときは、ヒト染色体上のα－サテライトＤＮＡ反復配列などの、内因性且つ構造的に変化のない染色体のセントロメアの上に正常に存在する反復ＤＮＡ配列をセントロメアが実質的に欠如していることを意味すると理解される。さらに、ネオセントロメアはゲノムの正常な非セントロメア領域から誘導されると考えられる。

【００２０】

従って、本発明の一つの側面は、真核生物のネオセントロメアを規定するヌクレオチド配列を含む単離された核酸分子を提供する。

【００２１】

より具体的には、本発明は真核生物の染色体に由来するヌクレオチド配列を含み、且つその核酸分子が適合細胞中でトランケーションされた若しくは改変された形態で存在するネオセントロメア、又は適合細胞中に予め作成されたＤＮＡ実体としてトランスフェクションにより導入された場合複製でき、染色体外因子として作用できそして細胞分裂と共に分離できるネオセントロメアを包含する単離された核酸分子を提供する。

【００２２】

本発明はヒトのネオセントロメアの同定により本明細書に例示される。しかしながら、これは、本発明が哺乳動物、植物、鳥類、昆虫、ぜん虫、真菌、酵母及び爬虫類などの染色体由来の全ての真核生物のネオセントロメアに及ぶという理解の下でなされる。しかしながら、最も好ましいネオセントロメアは、ヒト染色体及びその哺乳動物相同染色体由来のものである。

【００２３】

本発明は、マーデル（１０）染色体のｑ' 腕の標的化されたテロメア関連トランケーション及びｐ' 腕の明らかな無作為トランケーションの組み合わせを用いた幾つかのネオセントロメアに基づくヒトのミニ染色体の構築に一部基づいている。マーデル（１０）染色体はヒト患者中で同定された染色体を指し、ヒト染色体１０の再配列から生ずる。このマーデル（１０）マーカーは有糸分裂において安定であり、本発明によれば非セントロメアと見られる位置に機能性のネオセントロメアを含む。マーデル（１０）のネオセントロメアは染色体１０のｑ２４とｑ２６の間に位置し、より具体的には　ｑ２５付近に位置する。さらに具体的に言えば、このネオセントロメアは染色体１０のｑ２５．２に位置する。本発明はマーデル（１０）染色体のｑ２４～ｑ２６領域からクローニングされたＤＮＡ並びに正常なヒト染色体１０の上の対応する領域により例示される。これらのＤＮＡ分子は機能を有するネオセントロメアを含む。しかしながら、本発明は哺乳動物及び非哺乳動物並びに植物、昆虫、ぜん虫、真菌、爬虫類及び酵母などの任意の染色体の上の任意のネオセントロメアに及ぶ。

【００２４】

１０ｑ２５領域由来のコスミドプローブ及びＢＡＣプローブを用いて詳細に地図化すると、トランケーション部位を定めることができ、ＮＣ－ＭｉＣｓがこの領域由来の単一コピーの無傷のＤＮＡを含むことを証明することができる。パン－α－サテライトＤＮＡを用いた広範なＦＩＳＨ、全ヒト染色体の全染色体ペイント、及び異なる部分染色体－１０のペイントにより、ＮＣ－ＭｉＣｓのいずれもがα－サテライトＤＮＡ又は他のヒトゲノム配列の検出可能な量を獲得しなかったことが明らかにされた。地図化データに基づいて、ＮＣ－ＭｉＣｓ３、４及び５のサイズは、それぞれ約1.6 、1.6 及び0.8 Ｍｂであると見積もられた。

【００２５】

本発明はさらに、ヒトのネオセントロメア又はその機能性誘導体又はその潜在型、合成型若しくはハイブリッド型、又はその哺乳動物若しくは非哺乳動物の相同染色体を規定する三次元構造を有する核酸分子又はその化学的等価物を意図する。

【００２６】

さらにより具体的には、本発明はヒトネオセントロメア又はその機能性誘導体又はその潜在型、合成型若しくはハイブリッド型又はその哺乳動物若しくは非哺乳動物の相同染色体を規定するコンホーメーションを指示するヌクレオチド配列又はその化学的等価物を有する単離された核酸分子であって、該セントロメアがＣＥＮＰ－Ａ及びＣＥＮＰ－Ｃ、これらのタンパク質に限定されないが、などのセントロメア結合性タンパク質と結合するものである核酸分子に関する。

【００２７】

本明細書においてセントロメアに関して「潜在的」と言う場合は、正常には機能しないがそれにもかかわらずある条件下では活性化されうるセントロメアへの言及をも含む。潜在的セントロメアは、それがα－サテライトＤＮＡ反復配列などの正常なセントロメアの上で見出される反復ＤＮＡ配列を実質的に含まない場合には、ネオセントロメアとみなしてもよい。非ヒト染色体中の好ましい反復ＤＮＡ配列はα－サテライトＤＮＡと機能的に等価な反復ＤＮＡであると考えられる。

【００２８】

従って、本発明の別の一側面はネオセントロメア又は機能性相同染色体を同定するための任意の方法であって、哺乳動物ＣＥＮＰ－Ａ及び／又はＣＥＮＰ－Ｃに特異的な抗体又は哺乳動物ＣＥＮＰ－Ａ及び／又はＣＥＮＰ－Ｃと交差相互作用できる抗体を用いるクロマチン免疫沈降法によりＤＮＡを単離する工程、免疫沈降法により単離されたＤＮＡを増幅する工程、増幅されたＤＮＡ中に標識を組み込む工程、及び次いで増幅されたＤＮＡを用いてゲノムＤＮＡ又はその等価物を含むＤＮＡアレーを精査する工程、及び該免疫沈降したＤＮＡにハイブリダイズするクローンを同定し単離する工程を含む方法の使用を意図する（５２）。

【００２９】

本発明のネオセントロメアのサイズは、約50ｂｐから約 2000 ｋｂｐまで、約70ｂｐから約 1000 ｋｂｐまで、約75ｂｐから約 800ｋｂｐまで、約80ｂｐから約 500ｋｂｐまで、約85ｂｐから約 200ｋｂｐまで、約90ｂｐから約100 ｋｂｐまで、約100 ｂｐから約 1ｋｂｐまで、約120 ｂｐから約 500ｂｐまで、約180 ｂｐから約 300ｂｐまでの範囲でありうる。一つの実施態様では、ネオセントロメアは約 60 ～100 kbp である。別の一実施態様ではネオセントロメアは約80ｋｂｐである。とりわけ好ましい実施態様では、ネオセントロメアは約 50 ｋｂｐから約 2000 ｋｂｐまでである。ネオセントロメアは、ＣＥＮＰ－Ａ結合ドメイン又は他のセントロメアタンパク質結合ドメイン、又は異なる複製タイミング、クロマチン構造、足場構造体、化学的修飾状態（例えば、アセチル化、メチル化、リン酸化、ポリＡＤＰリボシル化）を示すドメインなどの別種の構造的又は機能的に異なるドメインを包含してもよい。機能的に重要な全てのドメインを包含するためサイズが２０００ｋｂｐより大きなネオセントロメアも提供される。

【００３０】

本発明のミニ染色体のサイズは、約 500ｂｐから約 20000ｋｂｐまで、約 700ｂｐから約 1000 ｋｂｐまで、約 750ｂｐから約 8000 ｋｂｐまで、約 800ｂｐから約 5000 ｋｂｐまで、約 850ｂｐから約 2000 ｋｂｐまで、約 900ｂｐから約 1000 ｋｂｐまで、約 1000 ｂｐから約 10 ｋｂｐまで、約 1200 ｂｐから約 5000 ｋｂｐまで、約 1800 ｂｐから約 3000 ｋｂｐまでの範囲でありうる。一つの実施態様では、ミニ染色体は約 600～1000 ｋｂｐである。別の一実施態様では、ミニ染色体は約 800ｋｂｐである。とりわけ好ましい実施態様では、ネオセントロメアは約 500ｋｂｐから約 20000ｋｂｐまでである。ミニ染色体はネオセントロメア、複製起点及びテロメアなどの別種の構造的又は機能的に異なるドメインを包含してもよい。とりわけ好ましい実施態様では、ミニ染色体は活性なネオセントロメアを含んでいる。

【００３１】

本発明の核酸分子は、健常なヒト被験者由来の天然に生じるヌクレオチド配列を含んでもよく、あるいはマーデル（１０）染色体若しくは等価な状態若しくは類似の状態を与える染色体を保持する被験者などの一以上の染色体依存状態を示すヒト被験者由来のヌクレオ

チド配列を含んでもよく、又は天然に生ずる配列若しくは天然に生じない配列に関して一つ以上のヌクレオチド置換、欠失及び／又は付加を有してもよい。このような改変は本明細書では「誘導体」と呼ばれ、天然に生ずるヌクレオチド配列の突然変異、断片、部分、相同染色体及び類似体が含まれる。本発明の誘導体はなお機能性ネオセントロメアを規定していることが好ましい。

【００３２】

本明細書で「ネオセントロメア」と言う場合は、機能性ネオセントロメア又はその機能性誘導体への言及をも含み、ネオセントロメアが有糸細胞分裂の間における姉妹染色分体結合や染色体分離を容易にすることができ、及び／又はＣＥＮＰ－Ａ及び／又はＣＥＮＰ－Ｃ及び／又は他の機能的に重要なセントロメアタンパク質と結合することができ、及び／又は抗ＣＥＮＰ－Ａ抗体又は抗ＣＥＮＰ－Ｃ抗体又は他の機能的に重要なセントロメアタンパク質に対する抗体と相互作用できることを意味する。一般に、そして好ましくは、ネオセントロメアはＣＥＮＰ－Ｂ又は抗ＣＥＮＰ－Ｂ抗体と相互作用することはできない。また、ネオセントロメアは後成の機序若しくは他の関連機序により活性化され得る潜在的セントロメアであってもよい。ネオセントロメアはハイブリッド又は他のヒト、哺乳動物、植物又は酵母のネオセントロメアであってもよい。正確なコンホーメーションに達成するように、例えば、重合技術により提供された合成ネオセントロメアも本発明により意図される。ネオセントロメアのこのような形態及び定義の全てはこの用語の使用により包含される。

【００３３】

本発明の別の一側面は、ネオセントロメア若しくはその機能性誘導体若しくはその潜在型、合成型若しくはハイブリッド型を規定するコンホーメーションを指示するヌクレオチド配列若しくは化学的等価物を含む単離された核酸分子若しくは化学的等価物であって、該ネオセントロメアがα－サテライトＤＮＡなどの正常セントロメアの反復ＤＮＡを実質的に欠くものであり、ネオセントロメアがＣＥＮＰ－Ａ若しくはＣＥＮＰ－Ｃ若しくは他の機能的に重要なセントロメア結合性タンパク質又はそれらの抗体と結合できるものである核酸分子を提供する。

【００３４】

このネオセントロメアはＣＥＮＰ－Ｂ又はその抗体と相互作用できないことが好ましい。

【００３５】

一つの実施態様では、このネオセントロメアは、染色体１０上のｑ２４とｑ２６の間に位置し、特に染色体１０上のｑ２５に位置するヒトゲノム領域に対応する。

【００３６】

コンホーメーションを有するネオセントロメア若しくはその機能性誘導体若しくはその潜在型、合成型若しくはハイブリッド型を規定する本発明の核酸分子若しくはその化学的等価物は、とりわけヒトネオセントロメアに基づくミニ染色体（ＮＣ－ＭｉＣｓ）、ヒト人工染色体（ＨＡＣｓ）、哺乳動物人工染色体（ＭＡＣｓ）、酵母人工染色体（ＹＡＣｓ）及び植物人工染色体（ＰＬＡＣｓ）などのミニ染色体若しくは人工染色体の作成に有用である。ヒトＮＣ－ＭｉＣｓは、これらが大量のＤＮＡを収容でき、且つ、ヒトの細胞中で増殖できるので、特に有用である。このＮＣ－ＭｉＣｓはその起源が非ウイルス性であり、従って、通常のウイルスに基づくベクター系よりも、例えば治療用の遺伝子を導入することによる遺伝子治療に一層適している。さらに、ＮＣ－ＭｉＣｓは染色体外に留まっており、従って、挿入による又は置換による突然変異誘発の危険がない。ＮＣ－ＭｉＣｓの本質は細胞分裂のあいだ安定な分離を可能とするネオセントロメア若しくはその潜在型、合成型若しくはハイブリッド型の存在である。ＮＣ－ＭｉＣｓも染色体外に留まっており、従って、遺伝子治療に一層適している。「染色体外」と言うときは、それが主染色体と合体せず、事実上エピソームであることを意味する。

【００３７】

従って、本発明は真核細胞の複製起点、及びその中で遺伝子構築物が複製すべき細胞において機能を有するテロメアヌクレオチド配列により挟まれた真核生物のネオセントロメア

若しくはその機能的誘導体若しくはその潜在型、合成型若しくはハイブリッド型、又はその哺乳動物若しくは非哺乳動物の相同染色体を包含する核酸分子を含む遺伝子構築物であって、細胞内に導入されると該遺伝子構築物が細胞分裂と共に分離する環状若しくは線形形状いずれかの複製する染色体外因子となる遺伝子構築物を提供する。

【００３８】

より具体的には、本発明は、哺乳動物、ヒト、植物若しくは酵母の細胞の複製起点及び核酸分子を含むミニ染色体若しくは人工染色体の形態の遺伝子構築物をさらに意図する。ここで、核酸分子はテロメアヌクレオチド配列により挟まれた又はミニ染色体若しくは人工染色体がその中で複製すべき細胞中で機能するテロメア配列を殆ど又は全く持たない環状の、ヒトネオセントロメア若しくはその機能性誘導体若しくはその潜在型、合成型若しくはハイブリッド型又はその哺乳動物若しくは非哺乳動物の相同染色体を包含する。

【００３９】

別の一実施態様は、哺乳動物、ヒト、植物若しくは酵母の細胞の複製起点、及び核酸分子であって、テロメア配列により挟まれた、又はミニ染色体若しくは人工染色体がその中で複製すべき細胞中で機能するテロメア配列を殆ど又は全く持たない環状の、ヒトネオセントロメア若しくはその機能性誘導体若しくはその潜在型、合成型若しくはハイブリッド型又はその哺乳動物の相同染色体を規定する三次構造を有する核酸分子、を含むミニ染色体若しくは人工染色体の形態の遺伝子構築物を提供する。

【００４０】

さらに別の一実施態様は、哺乳動物、ヒト、植物若しくは酵母の細胞の複製起点、及びヒトネオセントロメアを規定するコンホーメーションを指示するヌクレオチド配列を有する核酸分子を含むミニ染色体若しくは人工染色体の形態の遺伝子構築物であって、上記セントロメアがＣＥＮＰ－Ａ及び／又はＣＥＮＰ－Ｃ及び／又は他のセントロメアタンパク質又はそれらの抗体と結合し、α－サテライトＤＮＡ反復配列を実質的に含まないものであり、上記核酸分子がテロメアヌクレオチド配列により挟まれているか又はミニ染色体若しくは人工染色体が複製すべき細胞の中で機能するテロメア配列を殆ど又は全く保持しない環状の形態である、遺伝子構築物に関する。

【００４１】

本発明はミニ染色体を作成する方法であって、

細胞中に、標的染色体のｑ’又はｐ’腕の一方若しくは他方の上にテロメア配列、選択可能マーカー、相同なターゲティングＤＮＡを有するベクターを含むトランケーション構築物を導入する工程、

該選択可能マーカーを発現する細胞を選択する工程、

該細胞に、該ｑ’又はｐ’を標的とするＤＮＡの他方を含む第２のトランケーション構築物を導入する工程、

該第２のトランケーション構築物と結合した選択可能マーカーを発現する細胞を選択する工程、及び

次いで、ネオセントロメアを含むトランケーションされた染色体を単離する工程、

を含む方法をさらに提供する。

【００４２】

一つの実施態様では、該ｑ’を標的とする腕及びｐ’を標的とする腕はマーデル（１０）染色体を標的とする。

【００４３】

特に好ましい実施態様では、ミニ染色体は図１に規定されているようにＮＣ－ＭｉＣ１～５と定義される。

【００４４】

本発明は、ネオセントロメアを挟んでいるｑ’及びｐ’腕を有する染色体を含む細胞をさらに提供する。ここで、ｑ’及びｐ’腕はその一方又は両方がトランケーションされている。

【００４５】

上述の方法又はその修正法は、発現のためなどの遺伝子若しくは他のヌクレオチド配列を導入するためにも使用されうる。

【００４６】

本発明は、ヒト、霊長類、昆虫、酵母の真核細胞又は本発明の遺伝子構築物を保持する他の真核細胞に及び、且つそれらから生産されるタンパク質に及ぶ。

【００４７】

本発明の遺伝子構築物は、ミニ染色体及び人工染色体並びにミニ染色体及び人工染色体の作成に有用なＤＮＡ構築物を含む。

【００４８】

該遺伝子構築物はマーカー遺伝子、ユニークな制限部位、又は外来のＤＮＡの挿入を促進するための組換え促進マーカー（例えば、Ｃｒｅリコンビナーゼと共に使用するためのＬｏｘＰ・ＤＮＡ）を含む。従って、本発明の遺伝子構築物は目的の生産物をコードする外来の又は異種のＤＮＡをさらに含む。目的の好ましい生産物は、サイトカイン類、受容体類、成長調節剤類などをコードする遺伝子などの薬学的に有用な遺伝子を含む。内因性遺伝子は野性型遺伝子又は改変された遺伝子で置換してもよい。

【００４９】

該遺伝子構築物中に固有に存在する特異的ＤＮＡ配列（例えば、推定的ＥＳＴｓ又は発現された遺伝子又は不安定ＤＮＡ因子）も、同定され、取り出され又はさらなる遺伝子操作若しくは遺伝子工学により改変されうる。他のＤＮＡ成分（例えば、治療用遺伝子若しくはマーカー遺伝子、ＬｏｘＰ・ＤＮＡ配列、Ｉ－ＳｃｅＩなどのユニーク制限部位）を上記遺伝子構築物に後で付加してその有用性又は適用を改良若しくは拡大してもよい。

【００５０】

外来の若しくは異種のＤＮＡは特定被験者中で十分な量が合成されない分子をコードしてもよい。従って、コピー数の増加によりより大量の分子の合成が可能となる。

【００５１】

従って、本発明は、複製起点、及びヒトのネオセントロメア若しくはその機能性誘導体若しくはその潜在型、合成型若しくはハイブリッド型又は哺乳動物若しくは非哺乳動物の相同染色体を定める第１の核酸分子、ペプチド、ポリペプチド又はタンパク質をコードする第２の核酸分子を含む遺伝子構築物であって、該第１及び第２の核酸分子がヒトテロメア配列により挟まれているか又は該遺伝子構築物がその中で複製すべき細胞中で機能するテロメア配列を殆ど又は全く保持しない環状の形態で存在するものである遺伝子構築物を意図する。

【００５２】

本明細書で「分離する」と言うときは、好ましくは有糸分裂的に安定な分離を意味する。安定な分離は、４～６か月の連続継代の後に娘細胞の４０～６０％より多くにミニ染色体若しくは人工染色体が存在する場合と決定するのが便利である。

【００５３】

本発明は、ＨＡＣｓ、ＭＡＣｓ及びＰＬＡＣｓなどの上述のＮＣ－ＭｉＣｓに類似の他のミニ染色体若しくは人工染色体に、又はミクロ染色体、合成染色体及びそれらの変異体などの他の記述若しくは名前による同様な実体に及ぶ。

【００５４】

本発明の別の一側面は、本発明のヒトネオセントロメア若しくはその哺乳動物及び非哺乳動物の相同染色体と結合し、相互作用し若しくはその他の方法で会合するペプチド、ポリペプチド及びタンパク質に関する。これらの分子は一次（１°）タンパク質と呼ばれるタンパク質であることが好ましい。この１°タンパク質はネオセントロメアに結合し、二次（２°）タンパク質はネオセントロメアと結合する前又は後に１°タンパク質に結合する。本発明のヒトネオセントロメアの同定は、例えば遺伝的障害の染色体をスクリーニングするために重要でありうる１°タンパク質及び２°タンパク質を検定するための機序を提供する。これは特に染色体分離の欠陥から生ずるダウン症候群における使用である。

【００５５】

１°タンパク質は、例えばゲルシフト検定により容易に検出される。ヒトネオセントロメアを規定する本発明の核酸分子を消化し、標識化し、１°タンパク質を含むと推定される核抽出物と接触させ、そしてゲル上で分離する。１°タンパク質はネオセントロメアの結合部分を保持する断片に結合すると、ＤＮＡ断片は結合したタンパク質のためより遅い速度でゲル中を移動する。

【００５６】

本発明は主題であるセントロメアと結合できる精製した１°タンパク質に及び、それをコードする遺伝子配列に及び、そしてそれに対する抗体にも及ぶ。

【００５７】

本発明のネオセントロメアは、例えば、ヒト線維肉腫細胞系統を用いて容易に同定され特性決定される。例えば、ネオセントロメアを保持すると疑われるＤＮＡは、一般的にはテロメア配列と共に線形形状で線維肉腫細胞中に導入される。次いで、人工染色体と呼ばれる、複製する染色体外の分離する因子の存在についてこの細胞をスクリーニングする。

【００５８】

本発明はさらに複製する染色体外の分離する核酸分子を保持する真核細胞を包含する。この真核細胞は哺乳動物細胞であることが好ましく、ヒト細胞であることが最も好ましい。本発明のこの側面の核酸分子が本明細書に記載されるように好ましい。

【００５９】

下記の細胞系統はポートン・ダウン、サリズベリ、ウィルツ、ＳＰ４　０ＪＧ、英国のＥＣＡＣＣ、センター・フォア・アプライド・マイクロバイオロジー・アンド・リサーチに２０００年１２月２０日に下記の受託番号の下に寄託された。

【００６０】

【表１】

細胞系統	アクロニン（Acronyn）	受託番号
CHO/BE ZB30	-	00122001
HT1080 MIC 1	NC-MiC1	00122002
HT1080-MIC 2	NC-MiC2	00122003
HT1080-MIC 3	NC-MiC3	00122004
HT1080-MIC 4	NC-MiC4	00122005
HT1080-MIC 5	NC-MiC5	00122006
HT1080-MIC 5a	NC-MiC5a	00122007
HT1080-MIC 5b	NC-MiC5b	00122008

【００６１】

本発明は下記の非限定的実施例によりさらに説明される。

【実施例１】

【００６２】

細胞培養及び化学物質

ＢＥ２Ｃ１－１８－５ｆ（５ｆと略称する）を先に記述したように（２７）培養した。ＨＴ１０８０及び誘導体を１０％ｖ／ｖウシ胎児血清（ＦＣＳ）を添加したＤＭＥＭ（ギブコＢＲＬ）中で培養した。培地にハイグロマイシン（ロッシュ）、ピューロマイシン（シグマ社）、又はゼオシン（インビトロゲン）を、それぞれ２５０μｇ／ｍｌ、１μｇ／ｍｌ、又は２００μｇ／ｍｌの濃度で添加した。培地に１００Ｕ／ｍｌのペニシリン及び１００ｍｇ／ｍｌのストレプトマイシン（ギブコＢＲＬ）を補充した。全ての細胞系統はサブコンフルエンスで維持した。微小細管解重合剤であるコルセミド（colcemid)(ギブコＢ

ＲＬ）又はノカダゾール（nocadazole)(シグマ）を細胞収穫の前にそれぞれ１０μＭの濃度で１時間又は０．１μｇ／ｍｌの濃度で６～１２時間培地に添加した。使用した化学物質は全て分子生物学的グレードのもので、市販のものを購入した。

【実施例２】

【００６３】

細胞系統のトランスフェクション

５ｆ及びＺＢ３０細胞系統のトランスフェクションは電気穿孔法を用いて行なった。簡単に述べると、10^7 個の対数増殖期の細胞を収穫し、ＰＢＳ中で２回洗浄し、８００μｌの電気穿孔用緩衝液（２０ｍＭヘペス，ｐＨ 7.0、１５０ｍＭのＮａＣｌ、５ｍＭのＫＣｌ、６ｍＭのグルコース、2.5 ｍＭのＮａＯＨ、１ｍＭのNa_2HPO_4）中に再懸濁した。0.4 ｃｍキューベット中で線形化したＤＮＡ２０μｇを細胞と混合し、バイオラド・ジーン・パルサー・エレクトロポレーターを用いて細胞を 1.2ｋＶ，２５μＦで電気穿孔した。ＨＴ１０８０細胞又は誘導体細胞のトランスフェクションは先に述べた（３０）ような電気穿孔法又はリポフェクションのいずれかを用いて行なった。リポフェクションのためには、細胞を、トランスフェクションの１日前に、１５０ｍｍプレートの上の４０ｍｌに$1～3×10^5$ 細胞／ｍｌで塗布した。これは実験の当日に５０～７０％コンフルエンシーをもたらした。２ｍｌの無血清ＤＭＥＭを用いて１００μｌのフジーン６トランスフェクション試薬（ベーリンガー・マンハイム）を希釈し、この懸濁液を３７℃で５分間インキュベートした。２０μｇのＤＮＡも２ｍｌの無血清ＤＭＥＭ中に希釈した。次いで、希釈されたフジーン６をＤＮＡ懸濁液中に滴下して添加した。この混合物を軽く叩き、室温で１５分間インキュベートした。インキュベーションの後、このＤＮＡ／フジーン６混合物を細胞の上に滴下して添加した。細胞を９６穴プレートに播種し、トランスフェクションの２４～４８時間後に選別を行なった。

【実施例３】

【００６４】

微小核体により媒介されるＨＴ１０８０への染色体移送

微小核体融合は先に記述した（３８）ように行なった。対数増殖期のドナーＺＢ３０細胞をコルセミド（１μｇ／ｍｌ）と一晩インキュベートした。４８時間後に細胞を収穫し、２０μｇ／ｍｌのサイトカラシンＢ（シグマ）を補充したパーコール／無血清ＤＭＥＭ（１：１）中に再懸濁した。次いで、細胞懸濁液をオークリッジ管（ナルジーン）に入れ３２℃で９０分間１８０００ｒｐｍで遠心分離に掛けた。細胞混合物の両方のバンドをペレットとし、無血清ＤＭＥＭで１回洗浄した後、３０、８、及び５μＭの等孔膜（ミリポア社，ＭＡ）を通して濾過した。次に微小核体を１０μｇ／ｍｌのＰＨＡＰ（ディフコ，ウェスト・モルシー，ＵＫ）を含む無血清ＤＭＥＭ中に再懸濁し、３７℃で４５分間受容体ＨＴ１０８０細胞と凝集させた。凝集の後、５０％ｗ／ｖＰＥＧ（ベーリンガー・マンハイム）を添加することにより細胞を融合させ、室温で２分間インキュベートした後無血清ＤＭＥＭで濯いだ。インキュベーションの後、１０％ｖ／ｖＦＣＳを含むＤＭＥＭ中で細胞を一晩培養した。次いで、その培地を２００μｇ／ｍｌのゼオシンを含むＤＭＥＭで置換し、細胞を１４日の期間選別しながら維持した、後コロニーをさらなる特性決定のために釣り上げた。

【実施例４】

【００６５】

ＦＩＳＨ／免疫蛍光

ＦＩＳＨ／免疫蛍光の結合は先に記述した（２５，５１）方法の改良法を用いて行なった。パン－α－サテライトプローブｐＴＲＡ７を用いるＦＩＳＨ及びテロメア配列（パースペクチブズ・バイオシステム，ＭＡ）のＰＮＡ－ＦＩＳＨは先に記述した（３９，４０）ように行なった。エピフルオレッセンス・ミクロスコピーは適当なフィルターセットを搭載したツァイス・アクソプランII（カール・ツァイス，カーネギー，オーストラリア）を用いて行なった。画像は、ソフトウェアIP Lab Version 2.5.5 (Scanalytics Inc., Fairfax, VA, USA) により制御されたパワーマックＧ３パーソナルコンピュータに連結した冷

却電荷結合素子ビデオカメラ（SenSys 2, Photometrics, Tuscon, AZ, USA)を用いてデジタルで収録した。染色体ペインティング実験はＷＣＰ染色体ペイントキット（Vysis Inc.）を用いて製造者の説明書に従って行なった。部分染色体－１０（subchromosome-10）ペイントは、Ｍ．ロッキ（M. Rocchi)（ユニバーシティ・オブ・バリ）から得た体細胞放射ハイブリッドゲノムＤＮＡから誘導した。体細胞ハイブリッドＤＮＡのインターーアル・アンプリフィケーション（inter-Alu amplification)は、先に記述した（４１）ように、プライマー５’ＧＧＡＴＴＡＣＡＧＧＹＲＴＧＡＧＣＣＡ（配列番号：１）及び５’ＲＣＣＡＹＴＧＣＡＣＴＣＣＡＧＣＣＴＧ（配列番号：２）を用いて行なった。ペイントプローブ及びＦＩＳＨの標識化は標準的方法を用いて行なった。

【００６６】

ポリクローナル抗ＣＥＮＰ－Ａ、モノクローナル抗ＣＥＮＰ－Ｂ、ポリクローナル抗ＣＥＮＰ－Ｃ、及びＣＲＥＳＴ－６抗血清は先に記述した（４２，４３，４４，２７）。ポリクローナル抗ＣＥＮＰ－Ｅ（４５）、抗ＣＥＮＰ－Ｆ（４６）、及び抗ｈＢＵＢ１（４７）はＴ．Ｊ．イェン（Fox Chase Cancer Center)により、ポリクローナル抗ｈＺＷ１０（４８）はＢ．ウィリアムズ及びＭ．Ｌ．ゴールドバーク（コーネル・ユニバーシティ）により、そしてポリクローナルｐ５５ＣＤＣ（４９）はＪ．ワインシュタイン（Amgen 社）により、そしてポリクローナル抗ＴＲＦ１（３２）はチチアデランゲ（ロックフェラー・ユニバーシティ，ＮＹ）により提供された。抗血清ＣＲＥＳＴ－６は自己免疫ＣＲＥＳＴ病の患者由来のもので、ＣＥＮＰ－Ａ及びＣＥＮＰ－Ｂを含むセントロメア成分に対する抗体を含む（５１）。他の二次的抗体はジャクソン・イミュノリサーチ・ラボラトリー・インク（ウェストグローブ，ペンシルベニア，USA ）から購入した。

【実施例５】

【００６７】

トランケーション構築物

トランケーション構築物はｐＧＫ：ハイグロマイシン耐性遺伝子カセット、ｐＧＫ：ピューロマイシン耐性遺伝子カセット、又はｐＧＫ：ネオマイシン耐性遺伝子カセットのいずれかを含んでいた。ヒトテロメア反復の２ｋｂアレーは pBS Sal-tel(5) プラスミドから得られた（２８，５０）。マーデル（１０）のｐ’腕及びｑ’腕に対応するＤＮＡを含むゲノムコスミドクローンは先に記述された。高コピー反復ＤＮＡ配列を欠く５～１０ｋｂ断片（サザンハイブリダイゼーション後のＣＯＴ－１ハイブリダイゼーションの欠如により明らかである）は、標準的方法を用いて、両方の方向でトランケーションベクター中にサブクローニングした。または、ゲノムＤＮＡをトランケーションベクター中にクローニングするためロングレインジＰＣＲキット（ベーリンガー・マンハイム）を用いてＢＡＣクローンから直接ＰＣＲ増幅した。トランケーション構築物はすべてｐＡｌｔｅｒ（プロメガ社）ベクター骨格中で作成した。

【実施例６】

【００６８】

ゼオシン耐性マーカーのコンカテマー化（concatamerization)

ｐＺｅｏＳＶ２（＋）プラスミド（インビトロゲン）由来のゼオシン耐性カセットを、両側を挟むＮｏｔＩ制限部位を添加してＰＣＲ増幅した。これをｐＧＥＭ－Ｔ（プロメガ社）中にクローニングした。このプラスミド１００μｇをＮｏｔＩで消化し、フェノール／クロロホルム抽出により精製した。コンカテマー（鎖状体）を作成するため、消化されたＤＮＡ（総容量１００μｌ）に、１０μｌのリガーゼ緩衝液、４０ユニットのＴ４リガーゼ及び５μｌの１００ｍＭのＡＴＰを添加した。この結合反応は一晩行い、完了した反応液の１μｌをパルスフィールドゲル電気泳動により試験して生じたコンカテマーのサイズを求めた。ＤＮＡは、トランスフェクションの前に、フェノール／クロロホルム抽出し、エタノール沈殿した。

【実施例７】

【００６９】

ドットブロットハイブリダイゼーションを用いるｐ’腕トランケーションスクリーニング

２４穴プレート中の細胞をトリプシン処理により収穫し、真空下でハイボンドＮ＋（アマシャム社）を含むミニフォールド・ドット・ブロッティング・アパレイタス（９６パーブロット，シュレリヒェ・アンド・シュル・インク）に直接移した。移送後、ハイボンドＮ＋を１０分間変性し（１ＭのＮａＯＨ，１ＭのＮａＣｌ）、５分間で２回中和した（１Ｍトリス塩酸，1.5 ＭのＮａＣｌ）後、２×ＳＳＣ中で最終洗浄を行なった。次いで、膜を８０℃で 1.5時間焼き、標準的方法を用い、^{32}Ｐ標識化ｐＡｌｔｅｒベクター（プロメガ社）ＤＮＡを用いて精査した。

【実施例８】

【００７０】

マーデル（１０）標識化及びＨＴ１０８０細胞中への移送

マーデル（１０）染色体を含むＣＨＯに基づく体細胞ハイブリッド系統（５ｆと命名）（２７）を用いた。先に記述した（９，１２，２８）系に類似の系を用いて、最初のテロメア関連染色体トランケーション（ＴＡＣＴ）を図１Ａ（以下に述べる）に示すトランケーションベクターを用い５ｆに対して行なった。異なるトランケーションベクターでトランスフェクトされた２５０００個を超える薬剤耐性コロニーについての徹底的なスクリーニングによっても陽性のトランケーション事象は得られなかった。このことはこの５ｆ細胞系統がＴＡＣＴの適当な宿主でなかったことを示唆する。次いで、本発明者らはマーデル（１０）染色体をヒトＨＴ１０８０細胞中に移送することを決定した。何故なら、この細胞系統は相同組換えに優れていることが知られており（２９，３０）、テロメア活性を示し（３１，３２，３３）、そして微小核体により媒介される染色体移送の良好な受容体である（３４，３５）からである。ｐＧＥＭ－Ｔベクター中にクローニングされたゼオシン耐性遺伝子で５ｆ細胞中のマーデル（１０）を標識するため無作為挿入アプローチが最初に用いられた。染色体標識化についてのその後のＦＩＳＨスクリーニングを容易にするため、ゼオシン耐性遺伝子を含むZEO(登録商標)/pGEM-T構築物をコンカテマー化し、５０ｋｂより大きなＤＮＡ断片のみを用いて５ｆ細胞をトランスフェクトした。６３個の個々のゼオシン耐性コロニーをスクリーニングして、マーデル（１０）がその遠位のｑ’領域で標識化された１個の細胞系統（ＺＢ３０と命名）を同定した（図２Ａ）。この細胞系統は微小核体により媒介されるＨＴ１０８０細胞中への染色体移送におけるドナーとして使用された。６０個のゼオシン耐性微小核体融合コロニーのうちの１５個はマーデル（１０）を含むことが示された。ネオセントロメア領域由来のＢＡＣｓ、パン－α－サテライト、ＣＨＯゲノムＤＮＡ、及び抗セントロメア抗体の宿主を用いてこれらの細胞系統を広範なＦＩＳＨ分析及び免疫蛍光分析により、マーデル（１０）染色体及びそのネオセントロメアが無傷であり、染色体への如何なるα－サテライト反復又はＣＨＯ・ＤＮＡの組み込みも起こらなかったことが確認された。ＺＢＨＴ－１４と命名されたこれらの細胞系統の一つが、以後のトランケーション実験に用いられた。

【００７１】

無傷のマーデル（１０）を獲得したＺＢＨＴ－１４などの細胞系統に加え、この染色体の無作為にトランケーションされた誘導体を含む幾つかの細胞系統も融合生成物中に検出された。このような破損した染色体断片の形成及び移送は微小核体融合手順と関連する比較的普通の出来事であるように見えた（３６）。一つの細胞系統（ＮＣ－ＭｉＣ１と命名）は、それが先に位置付けられた１０ｑ２５ネオセントロメアドメイン（２７）の周りの約２ＭｂのＤＮＡを含むことがＦＩＳＨにより明らかにされたミニ染色体を保持していたので興味深かった（図１Ｃ）。この細胞系統はさらなる特性決定及びトランケーション研究のために保持された。

【実施例９】

【００７２】

トランケーション構築物

１０ｑ２５ネオセントロメア領域の約３Ｍｂをカバーする５０個以上のＢＡＣクローン及びコスミドクローンを含む完全な物理的地図が調製された（図１Ｃ）。この地図に基づいて、この領域由来の異なるターゲティングＤＮＡを含む幾つかのトランケーション構築物

が設計された。これらの構築物は、ハイグロマイシン耐性遺伝子（ｑ’腕トランケーション用）、ネオマイシン耐性遺伝子、又はピューロマイシン（ｐ’腕トランケーション用）耐性遺伝子のいずれか、末端にクローニングされたヒトテロメア配列、及びマーデル（１０）のｑ’腕又はｐ’腕のいずれか由来の５～１０ｋｂのターゲティングＤＮＡを含んでいた（図１Ａ）。最初のＴＡＣＴ実験はｑ’腕について行なわれ、その後ｐ’腕のトランケーションが行なわれた（図１Ｂ）。

【実施例１０】

【００７３】

ｑ’腕のトランケーション

ｑ’トランケーション実験にはＺＢＨＴ－１４細胞系統及びＮＣ－ＭｉＣ１細胞系統が用いられた。両細胞系統ともＹ３Ｃ９４コスミド（これはＢＡＣ・Ｂ７９Ｅ１６と重なり合う、図１Ｃ）由来の６ｋｂターゲティングＤＮＡを含む hyg(登録商標)(ハイグロマイシン耐性) トランケーション構築物でトランスフェクトされた。このターゲティング部位は抗セントロメア抗体結合性ＮＣドメインから約 0.2Ｍｂに位置している（２７）。

【００７４】

ＺＢＨＴ－１４細胞系統については、マーデル（１０）のｑ’トランケーションについての最初のスクリーニングが生じたハイグロマイシン耐性コロニーをゼオシン中に塗布することにより行なわれた。これは、マーデル（１０）のゼオシン含有染色体部分を喪失した細胞系統を同定させ、従って、ゼオシン感受性であった。総数７３００個のハイグロマイシン耐性コロニーから、２１０個がゼオシン感受性であることが示された。ＦＩＳＨ分析により、これらのコロニーの大部分が無作為トランケーション又は他の未知の再配列を含んでいることが明らかにされた。一つの細胞系統（ＮＣ－ＭｉＣ２と命名）は所望の標的化トランケーションを受けたように見え、さらに特性決定された。

【００７５】

１０ｑ２５ネオセントロメア領域に沿って位置付けられた既知のコスミドクローン又はＢＡＣクローンの宿主を用いたＮＣ－ＭｉＣ２のＦＩＳＨ分析により、ターゲティングＤＮＡの上又は近位のクローンが存在した（例えば、Ｙ３Ｃ９４、図２Ｂ）が、ターゲティングＤＮＡに遠位のクローンは全て（例えば、Ｙ３Ｃ１０９、図２Ｃ）存在しなかったことが明らかにされた（図１Ｃに要約されている）。トランケーションの部位をより正確に決定するため、意図されたターゲティング部位（図１Ｄ）の両側の直ぐ隣にある約１０ｋｂの３個のＰＣＲ断片（Ｆ１～Ｆ３）を調製し、ＦＩＳＨ分析に使用した。その結果はターゲティングＤＮＡの近くに位置した断片（Ｆ１）のみが陽性シグナルを与えた（図２Ｄ）が、二つの遠位の断片Ｆ２及びＦ３は両方とも陰性であった（図２Ｅ）。ＴＴＡＧＧＧテロメア反復ペプチド核酸（ＰＮＡ）プローブを用いたさらなるＦＩＳＨはトランケーションされた染色体の上のテロメア配列の種を証明した（図２Ｈ）。パン－α－サテライトプローブを用いる低度のストリンジェンシーのＦＩＳＨ及び抗ＣＥＮＰ－Ｂ抗体を用いる免疫蛍光（図２Ｆ）は、α－サテライトＤＮＡがトランケーションされた染色体の中に挿入されなかったことを確認した。これらのデータは、ＮＣ－ＭｉＣ２の生産の際に、マーデル（１０）のｑ’腕の大部分の除去をもたらす標的化されたトランケーション事象が起こることを強く支持する。

【００７６】

ＮＣ－ＭｉＣ２の有糸分裂時の安定性は、２０回以上の細胞分裂をハイグロマイシンの存在下又は非存在下で成長した細胞を比較することにより決定された。薬物選択の存在又は非存在下で成長した細胞について８５％という類似の保持割合が観察された。このことはＮＣ－ＭｉＣ２が有糸分裂の際安定であったことを示唆する。この染色体が機能性のネオセントロメアを維持するか否かさらに研究するため、ＣＲＥＳＴ－６自己免疫血清（２７）及びヒストンＨ３様タンパク質であるＣＥＮＰ－Ａ、ＣＥＮＰ－Ｃ及びキネシン（kinesin)様運動性タンパク質であるＣＥＮＰ－Ｅに対する特異的抗体を用いて免疫蛍光研究を行なった。ネオセントロメア（ＮＣ）ドメインに先に位置付けられたＥ８ＢＡＣ（３７）と同じ位置に位置付けられた強力な抗血清シグナルが試験された全てのタンパク質につい

て観察され（例えば、図２Ｇ）、これによりＮＣ－ＭｉＣ２の上でのネオセントロメア機能が証明された。

【００７７】

ＮＣ－ＭｉＣ１細胞系統を、Ｙ３Ｃ９４ＤＮＡ含有ｑ’トランケーション構築物でトランスフェクトすると、１０００を超えるハイグロマイシン耐性コロニーが生じた。これらは、標的化されたトランケーション事象の証拠である該構築物（図１Ａ及び実施例７を参照）中に含まれるベクターＤＮＡの喪失についてスクリーニングされた。得られた一つの細胞系統であるＮＣ－ＭｉＣ３をｑ’コスミド及びＢＡＣｓで、及びＰＣＲプローブであるＦ１～Ｆ３を用いて詳細なＦＩＳＨ分析を行なうと、意図されたＹ３Ｃ９４部位で正確に標的化されたトランケーションが起こったことが明らかにされた（ＦＩＳＨの結果の例は図３Ａ～Ｂに示し、図１Ｃに要約する）。さらなるｐ’の地図化及びＮＣ－ＭｉＣ３の分析は以下に述べる。

【実施例１１】

【００７８】

ｐ’腕のトランケーション

ＮＣ－ＭｉＣ２は、ピューロマイシン又はネオマイシンマーカーを保持する構築物及び３個の異なるｐ’領域由来のターゲティングＤＮＡ（図１Ｃ）を用いてさらなるトランケーションに付した。数回の独立したトランスフェクション実験により形成されたピューロマイシン耐性及びネオマイシン耐性コロニーを上記のようにベクターＤＮＡの喪失について精査することにより、並びにトランケーションされたＮＣ－ＭｉＣ２誘導体を同定するためＥ８・ＢＡＣ及び遠位のｐ’腕ＢＡＣ（Ｂ１０Ｋ１）（図１Ｂを参照）を用いる二重着色ＦＩＳＨにより、可能なターゲティングについてスクリーニングした。この分析は大部分のコロニーがＮＣ－ＭｉＣ２の意図され標的化された染色体トランケーションを含むようには見えないことを示した。しかしながら、ネオセントロメア抗原結合領域に比較的近いｐ’部位でトランケーションを示した二つの細胞系統（ＮＣ－ＭｉＣ４及びＮＣ－ＭｉＣ５）が同定された（２７）。これらはより詳細に特性決定された。

【実施例１２】

【００７９】

ＮＣ－ＭｉＣｓ３、４及び５の構造

図１Ｃは地図位置が既知のプローブを用いて行なわれたＮＣ－ＭｉＣｓ３、４及び５の詳細なＦＩＳＨ地図化の結果のまとめである（図３～５に実例の一部が示してある）。３個のＮＣ－ＭｉＣｓは全てＹ３Ｃ９４内に予想されたｑ’トランケーションを示した。ｐ’腕については、ＮＣ－ＭｉＣ３（及びその先祖であるＮＣ－ＭｉＣ１）、ＮＣ－ＭｉＣ４、及びＮＣ－ＭｉＣ５それぞれについて、染色体トランケーションはＢＡＣｓ・Ｙ１３Ｃ１２（存在）／Ｂ１７９Ｎ３（非存在）、Ｙ１３Ｃ１２（存在）／Ｂ４３Ａ１１（非存在）、及びＢＡ４８Ｌ２４（存在）／ＢＡ６９Ｋ１０（非存在）の間で見られた（図３Ｃ／Ｄ、４Ａ／Ｂ及び５Ａ／Ｂ）。異なるＮＣ－ＭｉＣｓの上での陽性ＦＩＳＨシグナルの強度は、試験されたコスミドプローブ及びＢＡＣプローブの全てについて、ＨＴ１０８０細胞における正常な染色体１０上で見られたものとは区別できなかった。このことは、ＮＣ－ＭｉＣｓの形成の途中ではＤＮＡの二本化（duplication)は起こらなかったことを示唆する。パン－α－サテライトＤＮＡプローブ（図３Ｅ、４Ｃ及び５Ｃ）を用いる低ストリンジェンシーＦＩＳＨハイブリダイゼーション及び抗ＣＥＮＰ－Ｂ抗体（図４Ｅ及び５Ｄ）を用いる免疫蛍光はセントロメアαサテライトＤＮＡの非存在を明らかにした。異なるＮＣ－ＭｉＣ細胞系統を、２４個のヒト染色体全てについて全染色体ペイントを用いたＦＩＳＨにより分析すると、染色体１０ペイントのみがＮＣ－ＭｉＣｓの上で陽性のシグナルを形成した。このことは、他の染色体由来のゲノムＤＮＡは検出可能な量でそれらの形成の間にＮＣ－ＭｉＣｓの上に転座しなかったことを示唆する（図６Ａ）。幾つかの部分染色体１０ペイントを用いるさらなる分析（位置については図１Ｂを参照）は、ＮＣ－ＭｉＣｓの上の非１０ｑ２５領域由来のＤＮＡが存在しないことを同様に証明した（図６Ｂ）。従って、ＮＣ－ＭｉＣｓ３、４及び５はそれぞれ１０ｑ２５ネオセントロメア領域か

らのみ由来した１個のコピーＤＮＡを含み、それらの全サイズは、それぞれ約 1.6、1.6 及び 0.8Ｍｂであると推定されると結論できる（図１Ｃ）。

【００８０】

高分子量ゲノムＤＮＡを異なる細胞系統から調製し、６ＭｂまでのＤＮＡを分離する種々の条件の下でパルス・フィールド・ゲル電気泳動にかけた。ＮＣ－ＭｉＣ３のみがゲルを移動した。このことは、これが線形のミニ染色体であることを示唆し、他のＮＣ－ＭｉＣｓは環状構造である可能性を提起する。酵母染色体マーカーとの比較から、ＮＣ－ＭｉＣ３については、ＰＦＧＥゲル上で、 1.6Ｍｂのサイズと推定された。パン－テロメアプローブを用いるＦＩＳＨ又はテロメア反復結合因子ＴＲＦ１に対する抗体を用いる免疫蛍光は、ＨＴ１０８０細胞中の正常染色体全てのテロメア末端上にシグナルを生じたが、線形のＮＣ－ＭｉＣ３（例えば、図４Ｄ）を含めＮＣ－ＭｉＣｓのいずれの上にも生じなかった。これは、テロメア配列の低レベルを検出することが技術的に不可能であること及び／又はＮＣ－ＭｉＣｓの環状性によるものであろう。

【実施例１３】

【００８１】

ＮＣ－ＭｉＣ安定性及びネオセントロメア活性

ＮＣ－ＭｉＣｓ３、４及び５の有糸分裂安定性は、選択の存在下又は非存在下で培養培地中での２０回以上の細胞分裂の後に検定した。ＮＣ－ＭｉＣｓを同定するためＢＡＣ・Ｅ８をＦＩＳＨ実験で用い、各細胞系統について１００個の細胞を評価した。ＮＣ－ＭｉＣ３とＮＣ－ＭｉＣ４の両者については、薬物選択の存在下又は非存在下で約８０％という類似の保持割合が観察された。これは、両染色体誘導体が選択圧の非存在下でさえも安定であったことを示唆する。選択の存在下でＮＣ－ＭｉＣ５細胞系統については３６％という保持割合が最初観察された。選択を除去した後、２０分裂の間培養すると、細胞の３７％がＮＣ－ＭｉＣ５を保持した。これは再び選択圧の非存在下での有糸分裂安定性を示唆する。ＮＣ－ＭｉＣｓ３及び４と比べ、ＮＣ－ＭｉＣ５の保持割合が減少したのは、この特定の細胞系統で見られた固有のゲノム不安定性と最も関係が深そうである。

【００８２】

ＮＣ－ＭｉＣ５は構築された最も小さな誘導体であるようであったので、そして元の細胞系統において骨格ゲノムの不安定性が観察されたので、本発明者らはこの系統をサブクローニングし、得られたクローン中におけるＮＣ－ＭｉＣ５の安定性を検討した。これらのサブクローン２個（ＮＣ－ＭｉＣ５ａ及びＮＣ－ＭｉＣ５ｂ）はＮＣ－ＭｉＣ５の安定性の大きな増加を示した。薬物選択の非存在下での５０回以上の細胞分裂の後、これら二つの細胞系統はそれぞれ９０％及び９３％の保持割合を示した。これらの保持割合は選択の存在下での同数の分裂を経た細胞中で見られたもの（それぞれ、９０％及び９１％）とは有意に異ならなかった。ＮＣ－ＭｉＣ５ａ細胞はミニ染色体を１コピー（細胞の７６％）か２コピー（細胞の１４％）かを含んでいたが、ＮＣ－ＭｉＣ５ｂ細胞では常に１コピーだけしか検出されなかった。ＮＣ－ＭｉＣ５ａ及び５ｂの構造は詳細なＦＩＳＨ分析により元のＮＣ－ＭｉＣ５染色体と同一であることが示された（図１Ｃ及び５）。

【００８３】

ＮＣ－ＭｉＣｓ上のネオセントロメアの機能の状態を研究するため免疫蛍光検出が用いられた。ＣＥＮＰ－Ａ、ＣＥＮＰ－Ｂ、ＣＥＮＰ－Ｃ、ＣＥＮＰ－Ｅ、ＣＥＮＰ－Ｆ、ｈＺＷ１０、ｐ５５ＣＤＣ及びＢＵＢ１を含むセントロメア関連タンパク質の宿主に対する抗血清を試験した。ＣＥＮＰ－Ｂを除く全てのタンパク質はＮＣ－ＭｉＣｓのそれぞれの上で明瞭に検出された（一部の例は図３Ｆ～Ｇ、４Ｆ～Ｈ、５Ｅ～Ｇに示す）。これらのタンパク質分布様相は、親のマーデル（１０）染色体で先に確立されたものと区別できなかった（２５，２７）。このことはＮＣ－ＭｉＣ誘導体がネオセントロメアの全活性を含むことを確認させる。

【実施例１４】

【００８４】

ＮＣ－ＭｉＣ２の細胞培養及びトランスフェクション

ＮＣ－ＭｉＣ２を、１０％ｖ／ｖＦＣＳを含むＤＭＥＭ（ギブコＢＲＬ）中で培養した。培地に２５０μｇ／ｍｌの濃度でハイグロマイシン（ロッシュ）を添加した。ＮＣ－ＭｉＣ２のトランスフェクションは電気穿孔法又はリポフェクションを用いて行なった。電気穿孔はバイオラド・ジーン・パルサー・エレクトロポレーターを用いて（0.4ｋＶ，２５０μＦ）行なった。リポフェクションのためには、トランスフェクションの１日前に細胞をプレートに塗布し、トランスフェクション時点で６０～７０％コンフルエンシーとなるようにした。希釈したフジーン６トランスフェクション試薬（２ｍｌの総量中の１００μｌは２０μｇのＤＮＡを含む）（ベーリンガー・マンハイム）の２ｍｌを細胞の上に滴下して添加した。トランスフェクションに使用したＤＮＡは、ヒトテロメア配列 htel 、ネオマイシン耐性遺伝子を挟む二つの loxP 部位、ターゲティングゲノムＤＮＡ、及びブラストサイジン耐性遺伝子を含むＴＡＣＴ構築物であった（図７Ａ）。抗生物質選択はトランスフェクションの２４～４８時間後に、２５０μｇ／ｍｌの濃度で、１４日の期間、適用された後、さらなる特性決定のためコロニーを釣り上げた。

【実施例１５】

【００８５】

ＮＣ－ＭｉＣ２のｐ’腕のトランケーション

ＴＡＣＴ実験は、Ｙ１３Ｃ１５コスミド／Ｂ１３７ｉｌ・ＢＡＣから誘導された４ｋｂのターゲティングＤＮＡ、ターゲティングＤＮＡの外側の二つの loxP 部位、ネオマイシン耐性遺伝子、及びブラストサイジン耐性遺伝子を含むネオマイシン耐性トランケーション構築物を用いて、ＮＣ－ＭｉＣ２細胞中でｐ’腕について行なわれた。ターゲティング事象の成功はブラストサイジン耐性遺伝子の喪失を生ずるであろう。１００００個のネオマイシン耐性細胞系統のうちで、約１０％がブラストサイジン感受性であった。ブラストサイジンへの感受性は該クローンをブラストサイジン５μｇ／ｍｌ中で培養することにより測定された。ネオマイシン耐性であるがブラストサイジン感受性であったクローンをＦＩＳＨ分析に掛けた。

【実施例１６】

【００８６】

ＮＣ－ＭｉＣ６の特性決定

一つの細胞系統（ＮＣ－ＭｉＣ６）が標的化されたトランケーションを受けたように見えたので、ＦＩＳＨにより広範に特性決定を行なった。ターゲティング部位に近くでコスミド及びＢＡＣｓが存在することが見出されたが、この部位から遠くのクローンは全て存在しなかった（図７Ｃに要約する）。本発明者らのＦＩＳＨ及び最近利用可能なゲノム配列データに基づいて、本発明者らはＮＣ－ＭｉＣ６のサイズが１．２Ｍｂであると推定した。

【００８７】

α－サテライト（ｐＴＲＡ７）及びＣＥＮＰ－Ｂへの結合はトランケーションされた染色体上では検出されなかった。ＣＲＥＳＴ－６自己免疫血清を用いた免疫蛍光法により、ＮＣ－ＭｉＣ６の上にネオセントロメア活性が確認された。従って、これらのデータは、標的化されたトランケーション事象がＮＣ－ＭｉＣ２中のマーデル（１０）のｐ’腕の大部分を除去したことを支持する。ＮＣ－ＭｉＣ６の有糸分裂安定性は、培養中選択の存在下及び非存在下で６０回分裂まで検定した。選択の非存在下では、２０回の細胞分裂後に８０％を超え、４０回の細胞分裂で７５％、そして６０回の分裂で７０％の保持割合が観察された（図８）。ネオマイシンの存在下では、４０回の分裂後にＮＣ－ＭｉＣ６の８５％が維持され、そして６０回を超える細胞分裂後にも７５％という高さが保持され、分裂当たりの損失割合は 0.42 ％であった。このことはＮＣ－ＭｉＣ６が有糸分裂において何回にもわたって安定であったことを示唆する。

【００８８】

二つの loxP 部位の存在はネオマイシン耐性遺伝子の切断及び CRE－リコンビナーゼを用いる特異的部位への新たな遺伝子の挿入のために使用されうる。遺伝子のこの切断及び挿入は、 CRE－リコンビナーゼ遺伝子を含むプラスミドの一過性トランスフェクション又は

CRE－リコンビナーゼのタンパク質トランスフェクションのいずれかを経て行なわれる。

【実施例１７】

【００８９】

ＧＦＰ（緑色蛍光タンパク質）を発現するマウス胚幹ＥＳ細胞へ及びマウスＦ９奇形癌細胞への微小核体により媒介される染色体移送

２０％ｖ／ｖＦＣＳを含むＥＳ培地（ギブコＢＲＬ）中でマウス胚幹細胞を培養した。マウスＦ９細胞は１０％ｖ／ｖＦＣＳを含むＤＭＥＭ（トレース・バイオサイエンシーズ）中で培養した。ＥＳ細胞のトランスフェクションは、バイオラド・ジーン・パルサー・エレクトロポレーターを用い、 0.8 kV 、３μＦで、電気穿孔法を用いて行なった。トランスフェクションに使用したＤＮＡは、緑色蛍光タンパク質をコードする遺伝子及びネオマイシン耐性遺伝子を含むｐＥＧＲＰ－Ｎ１（クローンテック）であった。抗生物質選択（２５０μｇ／ｍｌのＧ４１８ネオマイシン）は、トランスフェクションの２４～４８時間後に１４日の期間適用した後、コロニーを釣り上げ、スクリーニングのために大量培養した。

【００９０】

微小核体融合は先に記述したように行なった。４８時間コルセミド中で阻止された対数増殖期のドナーＺＢ３０細胞を、２０μｇ／ｍｌのサイトカラシンＢ（シグマ）を補充したパーコール／無血清ＤＭＥＭ（１：１）中に再懸濁した。次に、この細胞懸濁液を３２℃で９０分間１８０００ｒｐｍで遠心分離にかけた。細胞混合物の二つのバンドをペレット化し、無血清ＤＭＥＭで洗浄し、３０、８及び５μＭの等孔膜（ミリポア社，ＭＡ）を通して濾過した。次いで微小核体を５０％ｗ／ｖＰＥＧ（ロッシュ）を室温で２分間添加することにより受容体のネオマイシン耐性ＥＳ－ＧＦＰ細胞又はＦ９細胞と融合させた。インキュベーションの後、細胞を濯ぎ、２０％ｖ／ｖＦＣＳを含むＥＳ培地又は１０％ｖ／ｖＦＣＳを含むＤＭＥＭ中で一晩培養した後、２４時間後に抗生物質選択（ＥＳ細胞については２５０μｇ／ｍｌのＧ４１８ネオマイシン及び１００μｇ／ｍｌのゼオシン、そしてＦ９細胞については１００μｇ／ｍｌのゼオシン）を添加した。

【実施例１８】

【００９１】

ＥＳ－ＧＦＰｍａｒ（１０）＃１細胞系統及びＦ９－４－５ｍａｒ（１０）細胞系統の特性決定

ＥＳ－ＧＦＰｍａｒ（１０）＃１及びＦ９－４－５ｍａｒ（１）の広範なＦＩＳＨ分析により、マーデル（１０）染色体及びその中に含まれるネオセントロメアは無傷であったことが証明された。マウスのセントロメア／ペリセントロメアの主要及び微量サテライトＤＮＡ若しくはゲノムＤＮＡは両細胞系統のマーカー染色体上には検出されなかった。さらに、マーデル（１０）はこれらの細胞系統に存在する唯一のヒト染色体であった。マーデル（１０）の安定性は培養中の４５分裂まで選択の存在下で検定した。そしてマーカー染色体は両細胞系統において有糸分裂に際して安定であることを示した。

【００９２】

当業者は、本明細書で記載された発明が具体的に記述されたもの以外に変更や修正を受けうることを認めるであろう。本発明はこのような変更及び修正を全て含むことが理解されるべきである。また、本発明は本明細書で個々に又は集合的に言及され指摘された工程、特徴、組成物及び化合物、及び該工程又は特徴の任意の二以上のあらゆる組み合わせを全て含む。

【００９３】

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【図面の簡単な説明】
【００９４】
【図１Ａ】図１は、マーデル（１０）染色体からのネオセントロメアに基づくミニ染色体（ＮＣ－ＭｉＣと略す）形成を示す図式的表示である。
図１ＡはＴＡＣＴ標的に向かう構築物の構造を示す。マーデル（１０）の p' 又は q' 腕由来の標的に向かうＤＮＡを、哺乳動物の選択可能マーカー（ハイグロマイシン（登録商標）、ネオマイシン（登録商標）、又はピューロマイシン（登録商標））に隣接させて、クローニングされたヒトテロメアＤＮＡ（Ｈｔｅｌ）の小さなアレーを含むベクター中にクローニングした。構築物をベクターＤＮＡとテロメア反復の間の制限部位で線形化し末端にあるテロメア配列を露出させた。相同組換え事象の後に、このベクターＤＮＡを宿主染色体中に組み込むべきではない。ベクターＤＮＡの喪失は可能な組換え事象のスクリーニング検定法の設計を可能とする。
【図１Ｂ】図１Ｂはマーデル（１０）及びＮＣ－ＭｉＣｓの形成の図式を示す。白矢はマーデル（１０）が形成する際の正常な染色体１０上の切断点を示す。マーデル（１０）の長い腕及び短い腕はそれぞれ q' 及び p' と命名される。ＮＣ－ＭｉＣｓ１～５を生成させるトランケーション事象はＴｃ１及びＴｃ２により表される。マーデル（１０）に挿入されたゼオシン耐性遺伝子の位置は星印で示される。 p' 腕の喪失についてのスクリーニング検定で用いられたＢＡＣ・Ｂ１０Ｋ１の位置は示してある。染色体１０の上方の太い線はＮＣ－ＭｉＣｓの特性決定をするために用いられた部分染色体ＤＮＡペイントの位置及び命名を示す。
【図１ＣＤ】図１ＣはＮＣ－ＭｉＣｓの地図化。約３Ｍｂをカバーし、１０ｑ２５ネオセントロメア領域まで先に地図化された順序付けられたコスミド及びＢＡＣクローンが示してある。縦の影の部分はセントロメアタンパク質に結合するネオセントロメア（ＮＣ）ドメインを含むＥ８ＢＡＣを表す（２３，２７）。Ｅ８の左及び右への白矢じりは q' 及び p' トランケーション構築物を用いて標的化されたトランケーションの意図された位置を示す。（＋）はＮＣ－ＭｉＣに対するＢＡＣプローブ又はコスミドプローブについての陽性のＦＩＳＨ結果を示すが、（－）は陰性のＦＩＳＨ結果を示す。これらの異なるＮＣ－ＭｉＣｓのおおよそのサイズは括弧の中に示してある。
【００９５】
図１Ｄは標的化された q' トランケーション部位の特性決定を示す。 q' トランケーションを標的とするＤＮＡは、コスミドＹ３Ｃ９４（ＢＡＣ・Ｂ７９Ｅ１６中にも存在する）からサブクローニングされた６ｋｂＸｂａＩ断片であって斜線を引いたボックスで表される。Ｂ７９Ｅ１６由来のＰＣＲプローブの位置は二重頭の矢により示されＦ１、Ｆ２及びＦ３と命名される。これらのプローブのＮＣ－ＭｉＣｓ上でのハイブリダイゼーション状態は（＋）又は（－）で示され、 q' トランケーションが標的化事象の結果であったことを確認させる。
【図２】図２は、ＺＢ３０及びＮＣ－ＭｉＣ２のＦＩＳＨ分析及び／又は免疫蛍光分析の写真である。
【００９６】
（Ａ）ＺＢ３０のＦＩＳＨ分析はＥ８（緑色）及びマーデル（１０）（矢じり）の標識のゼオシン耐性遺伝子（赤色）とのハイブリダイゼーションを示す。
【００９７】
（Ｂ～Ｈ）ＮＣ－ＭｉＣ２についてのＦＩＳＨ分析及び／又は免疫蛍光分析。正常な染色体１０は矢じりにより示され、ＮＣ－ＭｉＣ２は矢によって示される。（ｉ ～ iv)それぞれ緑色、赤色、及びＤＡＰＩについての結合画像及び分離画像。
【００９８】
（Ｂ）Ｅ８プローブ（緑色）及びＹ３Ｃ９４コスミドプローブ（赤色）を用いたＦＩＳＨ分析であり、ＮＣ－ＭｉＣ２上にＹ３Ｃ９４が存在することを示す。
【００９９】
（Ｃ）Ｅ８（緑色）及び q' コスミドＹ３Ｃ１０９（赤色）を用いたＦＩＳＨ分析であっ

て、ＮＣ－ＭｉＣ２上にＹ３Ｃ１０９が存在しないことを示す。
【０１００】
（Ｄ）Ｅ８（緑色）及びＹ３Ｃ９４／Ｂ７９Ｅ１６に由来しｑ'を標的とするＤＮＡと重なり合う（図１Ｄを参照）ＰＣＲ断片Ｆ１（赤色）を用いたＦＩＳＨ分析であって、ＮＣ－ＭｉＣ２上にＦ１ＤＮＡが存在することを示す。
【０１０１】
（Ｅ）Ｅ８（緑色）及び標的に向かうＤＮＡ（図１Ｄ参照）に直ぐ遠位の領域にあるＢ７９Ｅ１６に由来するＰＣＲ断片Ｆ２（赤色）を用いたＦＩＳＨ分析であって、ＮＣ－ＭｉＣ２上にＦ２ＤＮＡが存在しないことを示す。
【０１０２】
（Ｆ）Ｅ８（緑色）を用いるＦＩＳＨ及び抗ＣＥＮＰ－Ｂ抗体（赤色）を用いた免疫蛍光分析であって、ＮＣ－ＭｉＣ２上にＣＥＮＰ－Ｂが存在しないことを示す。
【０１０３】
（Ｇ）Ｅ８（緑色）を用いるＦＩＳＨ及び抗ＣＥＮＰ－Ｅ抗体（赤色）を用いた免疫蛍光分析であって、ＮＣ－ＭｉＣ２上にＣＥＮＰ－Ｅタンパク質が存在することを示す。
【０１０４】
（Ｈ）Ｅ８（緑色）及びテロメア反復ＰＮＡプローブ（赤色）を用いたＦＩＳＨ分析であって、ＮＣ－ＭｉＣ２上のテロメア配列を示す。
【図３】図３はＮＣ－ＭｉＣ３についてのＦＩＳＨ分析及び／又は免疫蛍光分析の写真である。正常な染色体１０は矢じりによって示され、ＮＣ－ＭｉＣ３は矢によって示される。（ｉ～ ｉｖ)それぞれ緑色、赤色、及びＤＡＰＩについての結合画像及び分離画像。
【０１０５】
（Ａ）Ｅ８（緑色）及びｑ' ＢＡＣ・Ｂ７９Ｅ１６（赤色）を用いたＦＩＳＨ分析であって、ＮＣ－ＭｉＣ３上にＢ７９Ｅ１６が存在することを示す。
【０１０６】
（Ｂ）Ｅ８（緑色）及びｑ' コスミドＹ３Ｃ１０９（赤色）を用いたＦＩＳＨ分析であって、ＮＣ－ＭｉＣ３上にＹ３Ｃ１０９が存在しないことを示す。
【０１０７】
（Ｃ）Ｅ８（緑色）及びｐ’コスミドＹ１３Ｃ１２（赤色）を用いたＦＩＳＨ分析であって、ＮＣ－ＭｉＣ３上にＹ１３Ｃ１２が存在することを示す。
【０１０８】
（Ｄ）Ｅ８（緑色）及びｐ’ＢＡＣ・Ｂ１７９Ｎ３（赤色）を用いたＦＩＳＨ分析であって、ＮＣ－ＭｉＣ３上にＢ１７９Ｎ３が存在しないことを示す。
【０１０９】
（Ｅ）Ｅ８（緑色）及びパン－α－サテライトｐＴＲＡ－７プローブ（赤色）を用いたＦＩＳＨ分析であって、ＮＣ－ＭｉＣ３上にα－サテライトが存在しないことを示す。
【０１１０】
（Ｆ）Ｅ８（緑色）を用いたＦＩＳＨ分析及び抗ＣＥＮＰ－Ｅ抗体（赤色）を用いた免疫蛍光分析であって、ＮＣ－ＭｉＣ３上にＣＥＮＰ－Ｅが存在することを示す。
【０１１１】
（Ｇ）Ｅ８（緑色）を用いたＦＩＳＨ分析及び抗ＣＥＮＰ－Ｆ抗体（赤色）を用いた免疫蛍光分析であって、ＮＣ－ＭｉＣ３上にＣＥＮＰ－Ｆが存在することを示す。
【図４】図４は、ＮＣ－ＭｉＣ４のＦＩＳＨ分析及び／又は免疫蛍光分析の写真である。正常な染色体１０は矢じりにより、ＮＣ－ＭｉＣ４は矢により示される。（ｉ～ ｉｖ)それぞれ緑色、赤色及びＤＡＰＩについての結合画像及び分離画像。
【０１１２】
（Ａ）Ｅ８（緑色）及びｐ’コスミドＹ１３Ｃ１２（赤色）を用いたＦＩＳＨ分析であって、ＮＣ－ＭｉＣ４の上にＹ１３Ｃ１２が存在することを示す。
【０１１３】
（Ｂ）Ｅ８（緑色）及びｐ’ＢＡＣ・Ｂ４３Ａ１１（赤色）を用いたＦＩＳＨ分析であっ

て、ＮＣ－ＭｉＣ４の上にＢ４３Ａ１１が存在しないことを示す。
【０１１４】
（Ｃ）Ｅ８（緑色）及びパン－α－サテライトｐＴＲＡ－７プローブ（赤色）を用いたＦＩＳＨ分析であって、ＮＣ－ＭｉＣ４の上にα－サテライトが存在しないことを示す。
【０１１５】
（Ｄ）ＴＴＡＧＧＧテロメア反復ＰＮＡプローブを用いたＦＩＳＨ分析であって、全てのヒトテロメアの上で陽性シグナルを示すがＮＣ－ＭｉＣ４の上では示さない。
【０１１６】
（Ｅ）Ｅ８（緑色）を用いるＦＩＳＨ分析及び抗ＣＥＮＰ－Ｂ抗体（赤色）を用いた免疫蛍光分析であって、ＮＣ－ＭｉＣ４の上にＣＥＮＰ－Ｂが存在しないことを示す。
【０１１７】
（Ｆ）Ｅ８（緑色）を用いたＦＩＳＨ分析及び抗ＣＥＮＰ－Ｃ抗体（赤色）を用いた免疫蛍光分析であって、ＮＣ－ＭｉＣ４の上にＣＥＮＰ－Ｃが存在することを示す。
【０１１８】
（Ｇ）Ｅ８（緑色）を用いたＦＩＳＨ分析及び抗ＣＥＮＰ－Ｅ抗体（赤色）を用いた免疫蛍光分析であって、ＮＣ－ＭｉＣ４の上にＣＥＮＰ－Ｅが存在することを示す。
【０１１９】
（Ｈ）Ｅ８（緑色）を用いたＦＩＳＨ分析及び抗ＣＥＮＰ－Ｆ抗体（赤色）を用いた免疫蛍光分析であって、ＮＣ－ＭｉＣ４の上にＣＥＮＰ－Ｆが存在することを示す。
【図５】図５はＮＣ－ＭｉＣ５のＦＩＳＨ分析及び／又は免疫蛍光分析の写真である。正常な染色体１０は矢じりによりそしてＮＣ－ＭｉＣ５は矢により示される。（ｉ～ iv)それぞれ緑色、赤色、及びＤＡＰＩについての結合画像及び分離画像。
【０１２０】
（Ａ）Ｅ８（緑色）及びｐ’ＢＡＣ・ＢＡ４８Ｌ２４（赤色）を用いたＦＩＳＨ分析であって、ＮＣ－ＭｉＣ５の上にＢＡ４８Ｌ２４が存在することを示す。
（Ｂ）Ｅ８（緑色）及びｐ’ＢＡＣ・ＢＡ６９Ｋ１０（赤色）を用いたＦＩＳＨ分析であって、ＮＣ－ＭｉＣ５の上にＢＡ６９Ｋ１０が存在しないことを示す。
【０１２１】
（Ｃ）Ｅ８（緑色）及びパン－α－サテライトｐＴＲＡ－７プローブ（赤色）を用いたＦＩＳＨ分析であって、ＮＣ－ＭｉＣ５の上にα－サテライトが存在しないことを示す。
【０１２２】
（Ｄ）Ｅ８（緑色）を用いたＦＩＳＨ分析、及び抗ＣＥＮＰ－Ｂ抗体（赤色）を用いた免疫蛍光分析であって、ＮＣ－ＭｉＣ５の上にＣＥＮＰ－Ｂが存在しないことを示す。
【０１２３】
（Ｅ）Ｅ８（緑色）を用いたＦＩＳＨ分析、及び抗ＣＥＮＰ－Ａ抗体（赤色）を用いた免疫蛍光分析であって、ＮＣ－ＭｉＣ５の上にＣＥＮＰ－Ａが存在することを示す。
【０１２４】
（Ｆ）Ｅ８（緑色）を用いたＦＩＳＨ分析、及び抗ＣＥＮＰ－Ｃ抗体（赤色）を用いた免疫蛍光分析であって、ＮＣ－ＭｉＣ５の上にＣＥＮＰ－Ｃが存在することを示す。
【０１２５】
（Ｇ）Ｅ８（緑色）を用いたＦＩＳＨ分析、及び抗ＣＥＮＰ－Ｅ抗体（赤色）を用いた免疫蛍光分析であって、ＮＣ－ＭｉＣ５の上にＣＥＮＰ－Ｅが存在することを示す。
【図６Ａ】図６はＮＣ－ＭｉＣｓの染色体ペインティング分析の写真である。
【０１２６】
図６Ａは２４のヒト染色体（１～２２，Ｘ，Ｙ）全てについての全染色体ペイントを示す。左側のパネルは、近点ヘテロ染色体領域を除く正常なヒト染色体の上の陽性ペインティング（緑色）、及び末端動原体の染色体の短腕及びＹのｑ１２領域の上の陽性ペインティングを示す。上方右パネルはＮＣ－ＭｉＣ４の上の対応するペインティングの結果を示すが、下方の右パネルはＮＣ－ＭｉＣ５の結果を示す。染色体ペイントは染色体１０を除き全て、重なりペイント（緑色）及びＥ８（赤色）シグナルが欠如していることから明らか

なように（示していない分離個別画像により確認された）ＮＣ－ＭｉＣｓの上では陰性であるが、染色体１０のペイントシグナルはＮＣ－ＭｉＣｓの上で明らかに見られる。これは、結合黄色を生ずる赤と緑のシグナルの重なりにより明らかである。

【図６Ｂ】図６ＢはＮＣ－ＭｉＣ５の上での部分染色体－１０のペイントを示す。染色体１０の上でのこれらの部分染色体ペイントの位置は図１Ｂに図式的に示してある。左手のパネルはこれらのペイントの名前及び正常な染色体１０の上でのＦＩＳＨの結果（緑色）を示す。右手のパネルはＮＣ－ＭｉＣ５の上でのこれらのペイントについて得られた陰性の結果を示す。Ｅ８シグナルは赤色である。染色体及びＮＣ－ＭｉＣｓはスケールに示していない。

【図７Ａ】図７はＮＣ－ＭｉＣ２のトランケーションを経由するＮＣ－ＭｉＣ６構築を示す図式的表示である。

【０１２７】

図７ＡはＮＣ－ＭｉＣ２から p' 腕をトランケーションするために使用した標的に向かうＴＡＣＴ構築物の構造を示す。マーデル（１０）の p' 腕から得た標的に向かうＤＮＡ及び哺乳動物の選択可能マーカー（ブラストサイジン耐性遺伝子，blasticidin(登録商標)）を、抗生物質耐性遺伝子，neomycin（登録商標）に隣接したクローニングされたヒトテロメアＤＮＡ（Ｈｔｅｌ）の小さなアレーを含むベクター中にクローニングした。構築物をベクターＤＮＡとテロメア反復の間の制限部位で線形化して末端のテロメア配列を露出させた。相同組換え事象の後では、該ブラストサイジン耐性遺伝子は宿主染色体中に組み込まれてはならない。ブラストサイジン耐性遺伝子の喪失により、可能な組換え事象についてのスクリーニング検定を設計することが可能となる。

【図７Ｂ】図７Ｂはマーデル（１０）及びＮＣ－ＭｉＣｓの図式的形成を示す。白矢は、マーデル（１０）を作成する際の正常染色体１０の上の切断点を示す。マーデル（１０）の長腕及び短腕はそれぞれ q' 及び p' と命名する。ＮＣ－ＭｉＣ２はマーデル（１０）から（先に記述したように）Ｂ７９ｅ１６／Ｙ３Ｃ９４でトランケーションされた（Ｔｃ１）のに対し、ＮＣ－ＭｉＣ６は、（Ａ）で記述されたＴＡＣＴ構築物を用いたＢ１３７ｉＬ／Ｙ１３ｃ１５におけるトランケーション２，Ｔｃ２の結果である。

【図７Ｃ】図７ＣははＮＣ－ＭｉＣｓ２及び６の地図化である。約３Ｍｂをカバーし、１０ｑ２５ネオセントロメア領域まで先に地図化された順序付けられたコスミドクローン及びＢＡＣクローンが示してある。垂直の影を付した領域はセントロメアタンパク質ＣＥＮＰ－Ａに結合するドメインを表す（５１）。白矢じりは標的化されたトランケーションの位置を示す。（＋）はＮＣ－ＭｉＣの上でのＢＡＣプローブ若しくはコスミドプローブについての陽性のＦＩＳＨ結果を示すが、（－）は陰性のＦＩＳＨ結果を示す。異なるＮＣ－ＭｉＣｓのサイズの概略は括弧内に示してある。

【図８】図８は、１５０μｇ／ｍｌの濃度のネオマイシン薬Ｇ４１８の存在下若しくは非存在下のいずれかで６０分裂の間ＮＣ－ＭｉＣ６を培養した後、安定性を試験するため種々の間隔でそれを収穫した場合の結果を示す表及び図式的表示である。そのＸ軸は分裂の数を表し、Ｙ軸はＦＩＳＨ分析で求められた培養中に存在するＮＣ－ＭｉＣのパーセンテージを表す。その結果はＧ４１８の存在下又は非存在下におけるＮＣ－ＭｉＣ６の有糸分裂安定性を示した。

【図１Ａ】

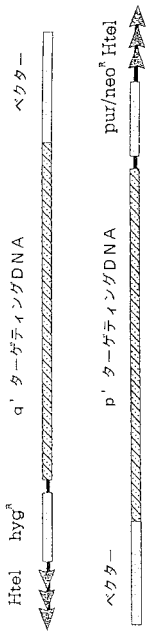

Figure 1A

【図１Ｂ】

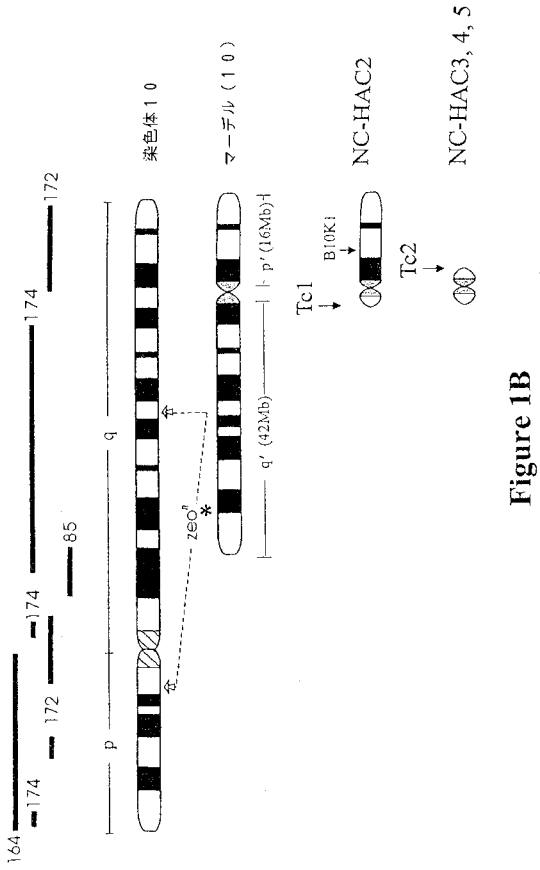

Figure 1B

【図１ＣＤ】

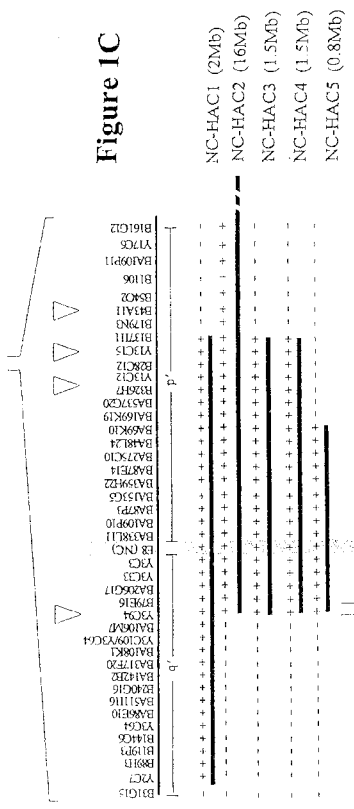

Figure 1C

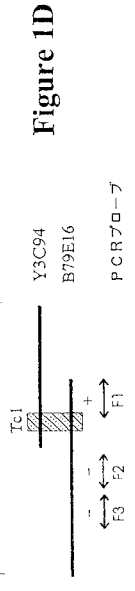

Figure 1D

【図２】

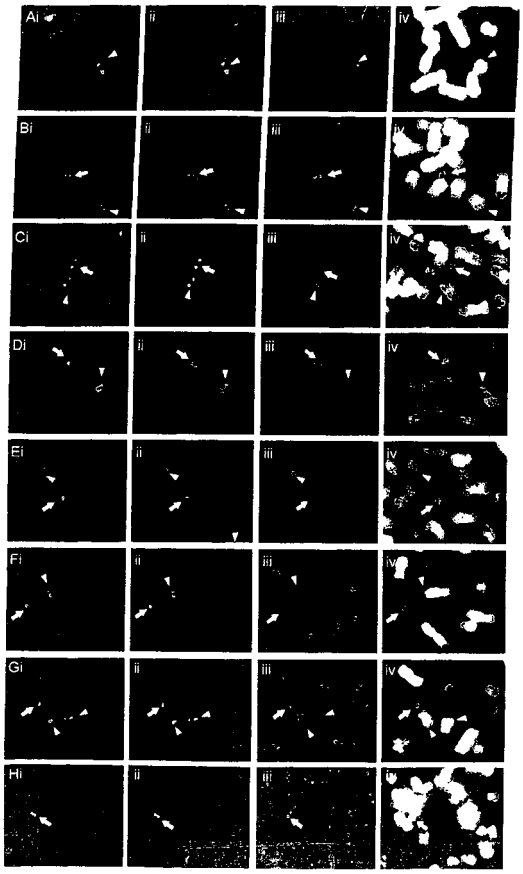

Figure 2

【図３】

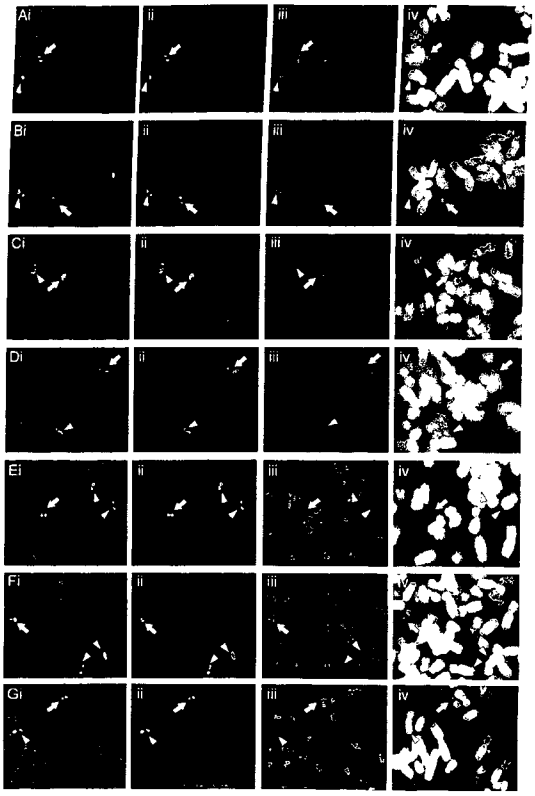

Figure 3

【図４】

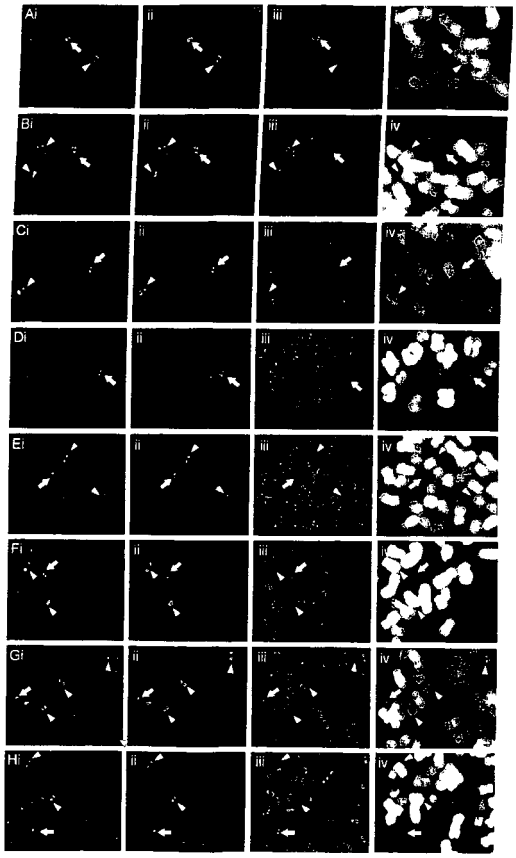

Figure 4

【図５】

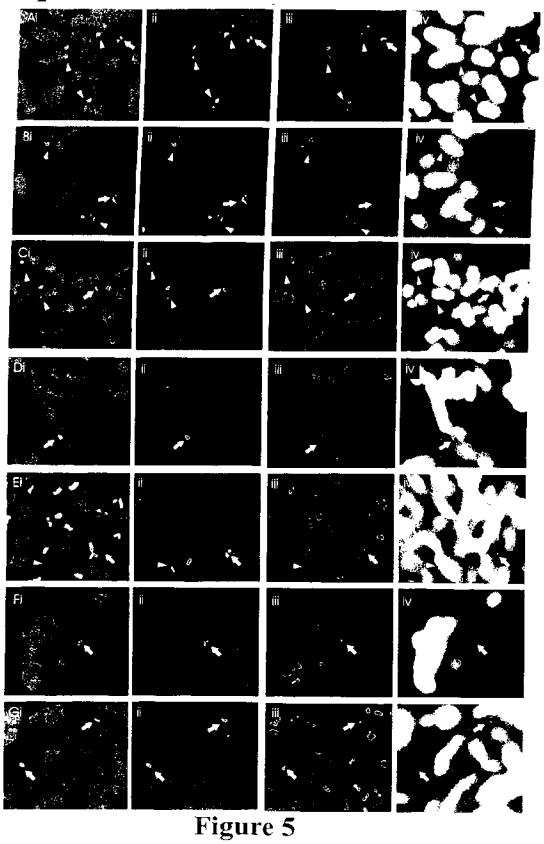

Figure 5

【図６Ａ】

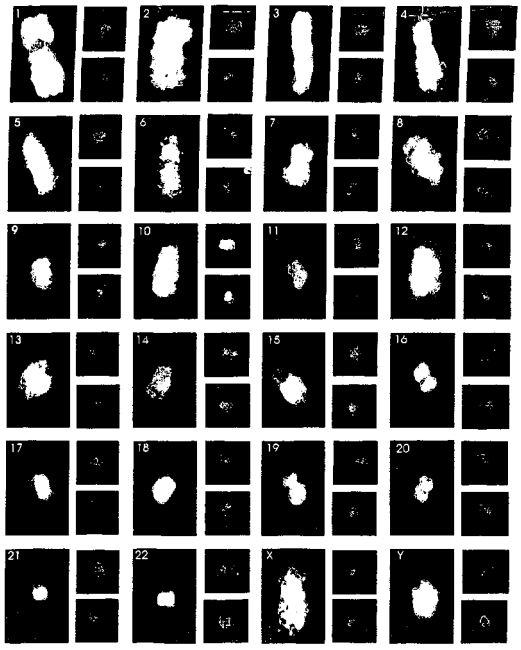

Figure 6A

【図６Ｂ】

Figure 6B

【図７Ａ】

Figure 7A

【図７Ｂ】

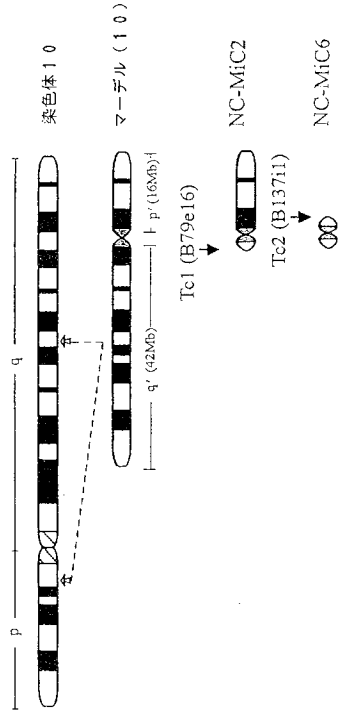

Figure 7B

【図７Ｃ】

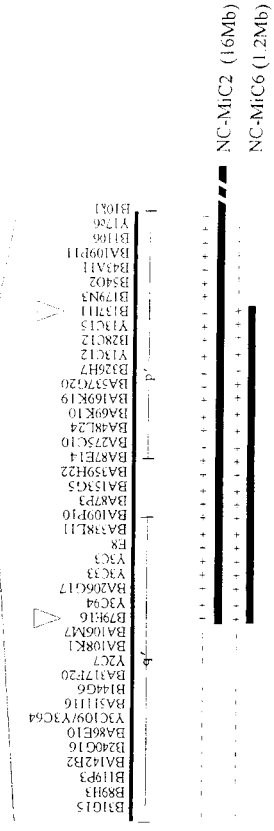

Figure 7C

【図８】

細胞系統	継代数	分裂数	薬物選択	評価された細胞の数	保持のパーセンテージ	分裂当たりの損失
MIC-6	5	10	ネオマイシン	20	19/20 (95%)	0.50%
			なし	20	18/20 (90%)	1.00%
	10	20	ネオマイシン	20	17/20 (85%)	0.75%
			なし	20	16/20 (80%)	1.0%
	20	40	ネオマイシン	20	16/20 (80%)	0.5%
			なし	20	15/20 (75%)	0.625%
	30	60	ネオマイシン	20	15/20(75%)	0.42%
			なし	20	14/20(70%)	0.5%

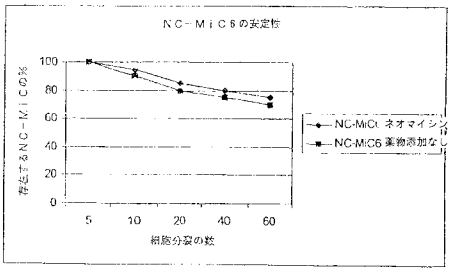

Figure 8

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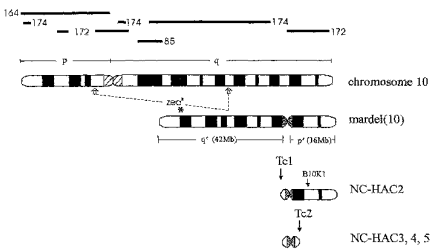
[Continued on next page]

(54) Title: NEOCENTROMERE-BASED MINI-CHROMOSOMES OR ARTIFICIAL CHROMOSOMES



(57) Abstract: The present invention is directed generally to a defined or isolated nucleic acid molecule encompassing a neocentromere or a functional derivative thereof or a latent, synthetic or hybrid form thereof and its use *inter alia* in developing a range of eukaryotic mini-chromosomes and artificial chromosomes including mammalian (e.g. human) and non-mammalian mini-chromosomes and artificial chromosomes. The present invention further provides a telomere-associated chromosome truncation (TACT) approach to develop mini-chromosomes, but is not limited to this approach. It is directed to any truncation approach that produces a neocentromere-containing mini-chromosome, or any transfection approach using cloned or pre-fabricated DNA that provides neocentromere function in the construction of artificial chromosome. Such mini-chromosomes and artificial chromosomes are useful in a range of genetic therapies.

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NEOCENTROMERE-BASED MINI-CHROMOSOMES OR ARTIFICIAL CHROMOSOMES

FIELD OF THE INVENTION

The present invention is directed generally to a defined or isolated nucleic acid molecule encompassing a neocentreomere or a functional derivative thereof or a latent, synthetic or hybrid form thereof and its use *inter alia* in developing a range of eukaryotic mini-chromosomes and artificial chromosomes including mammalian (e.g. human) and non-mammalian mini-chromosomes and artificial chromosomes. The present invention further provides a telomere-associated chromosome truncation (TACT) approach to develop mini-chromosomes, but is not limited to this approach. It is directed to any truncation approach that produces a neocentromere-containing mini-chromosome, or any transfection approach using cloned or pre-fabricated DNA that provides neocentromere function in the construction of artificial chromosome. Such mini-chromosomes and artificial chromosomes are useful in a range of genetic therapies.

BACKGROUND OF THE INVENTION

Bibliographic details of the publications numerically referred to in this specification are collected at the end of the description.

Reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in Australia or any other country.

The rapidly increasing sophistication of recombinant DNA technology is greatly facilitating research and development in the medical and allied health fields. A particularly important area is in mammalian including human genetics and the elucidation of the molecular mechanisms behind genetic abnormalities. Progress in research in this area has been hampered by the lack of a fully sequenced nucleic acid molecule containing a human

centromere. The identification and/or cloning of such a molecule would promote the development of techniques for introducing genes into eukaryotic cells and in particular mammalian including human cells and will be an important asset to gene therapy and the development of expression systems for the production of a range of recombinant gene products in mammalian cells. Importantly, the identification and/or cloning of a fully sequenced centromeres facilitates the development of mammalian mini-chromosomes and artificial chromosomes.

Mammalian mini-chromosomes and artificial chromosomes have a variety of potential biotechnological and therapeutic applications arising from their ability to exist episomally and allow expression of genes under their endogenous control elements independently of the host genomic DNA. Because they are in effect fully functional mammalian chromosomes, there is no theoretically upper limit to the size of DNA that can be introduced into these entities. By analogy with their yeast counterparts, it has been assumed that mammalian mini-chromosomes and artificial chromosomes require a functional mammalian centromere, telomeres and DNA replication origins in order for proper segregation. At present, the least understood and most complex of these three components is the centromere.

The identification of an increasing number of protein components necessary for correct centromere activity, and the characterization of centromere DNA sequences in a variety of species, have greatly increased the knowledge of the mechanisms underlying centromere formation and function (1,2,3). This knowledge has facilitated the development of a number of strategies for mammalian mini-chromosome and artificial chromosome construction. One strategy involves the *de novo* formation of human artificial chromosomes by co-transfection of telomeric DNA with large arrays of human α-satellite into human cells (4,5,6,7). Studies using this strategy have shown that only α-satellite DNA-containing CENP-B boxes can participate in *de novo* artificial chromosome formation (5,8). While some of the generated artificial chromosomes were linear in structure (4), others were consistently circular (5,7,8). The artificial chromosomes ranged in size from ~1-13 Mb and were typically one or more orders of magnitude larger than the

input DNA. This increase in size has been attributed to end-joining of input DNA following transfection (4).

A different strategy involves the use of telomere-associated chromosome truncation to remove non-essential chromosomal materials around a normal centromere to produce a mini-chromosome *in situ*. Sequential truncation of a human X chromosome has yielded a 2.5-Mb mini-chromosome comprising approximately 1.8 Mb of X-chromosome α-satellite DNA and 400 kb of proximal Xp DNA (9,10,11). This chromosome shows mitotic stability comparable to that of the normal human X chromosome (10,11). A similar approach has produced a number of human Y chromosome-derived mini-chromosomes, ranging in size from ~0.7 Mb to over 4Mb, with the smaller ones being relatively unstable (12,13). The larger mini-chromosomes were stably maintained in CHO cells, chicken DT40 cells and mouse L cells but showed poor stability when introduced into mouse ES cells, suggesting differential requirements for correct centromere function in different cell types (14,15).

A third strategy for production of mammalian artificial chromosomes involves the amplification of pericentric DNA followed by controlled breakage of chromosomes to produce satellite DNA-based artificial chromosomes of between 60 and 400 Mb (16,17,18,19,20).

In recent years, neocentromeres (NCs) that lack the repeat sequences traditionally associated with centromere function have been described in humans (21) and *Drosophila* (22). Characterization of the underlying DNA and protein-binding profile of neocentromeres in humans has suggested epigenetic mechanism of centromere formation independent of primary DNA sequence composition (23,24,25,26). The discovery of neocentromeres provides an alternative approach to the construction of mini-chromosomes and artificial chromosomes to those previously described that have been based on the use of repeated centromere DNA sequences.

In accordance with the present invention, the inventors have generated a series of

mitotically stable, human mini-chromosomes containing a fully functional human neocentromere. The generation of a human mini-chromosome permits the development of genetic therapies, transgenic plant and animal production and recombinant protein production.

SUMMARY OF THE INVENTION

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

Nucleotide and amino acid sequences are referred to by a sequence identifier number (SEQ ID NO:). The SEQ ID NOs: correspond numerically to the sequence identifiers <400>1, <400>2, etc. A sequence listing is provided after the claims.

The present invention is predicated in part on the use of a telomere-associated chromosome truncation (TACT) approach to develop mini-chromosomes. Truncation constructs are developed comprising a selectable marker, a targeting DNA sequence homologous to a small region on the p' or q' arms of a eukaryotic chromosome such as human mardel(10), and a small array of telomeric sequences. p' and q' arms refer to the short and long arms of the mardel(10) chromosome but also refer generally to the short and long arms of any marker chromosome containing a neocentromere. A first truncation construct is transfected into a target cell and the marker selected. This results in a target chromosome with a truncated p' or q' arm depending on the truncation construct used. A second truncation construct is then transfected into the same cell employing the other of the p' or q' arm truncation construct. Again, following selection, a truncated form of the p' or q' arm of the target chromosome is obtained. The resulting mini-chromosome may then be isolated and used for gene therapy or gene expression. A modified targeting method that may or may not include a telomere DNA may also be used to introduce genes or other nucleotide sequences into a target chromosome.

One aspect of the present invention provides an isolated nucleic acid molecule comprising a sequence of nucleotides which defines an eukaryotic neocentromere.

More particularly, the present invention provides an isolated nucleic acid molecule

comprising a sequence of nucleotides derived from a eukaryotic chromosome and encompassing a neocentromere which nucleic acid molecule exists in a truncated or modified form in a compatible cell or, when introduced by transfection as a pre-fabricated DNA entity into a compatible cell, is capable of replicating, acting as an extra chromosomal element and segregating with cell division.

Another aspect of the present invention contemplates the use of a method for identifying a neocentromere or a functional homolog, said method comprising isolating DNA by chromatin immunoprecipitation using an antibody specific to mammalian CENP-A and/or CENP-C or an antibody capable of cross interacting with mammalian CENP-A and/or CENP-C, amplifying the DNA isolated by immunoprecipitation and incorporating label into the amplified DNA and then using the amplified DNA to probe a DNA array comprising genomic DNA or its equivalent and identifying and isolating clones which hybridize to said immunoprecipitated DNA.

A further aspect of the present invention provides an isolated nucleic acid molecule in the form of a human neocentromere-based mini-chromosome (NC-MiCs) comprising a neocentromere or a latent, synthetic or hybrid form thereof which enable stable segregation during cell division.

Yet another aspect of the present invention provides a method for generating a mini-chromosome, said method comprising:-

introducing into a human or mammalian cell which carries a chromosome containing a neocentromere, a truncation construct comprising a vector having telomeric sequences, a selectable marker, homologous targeting DNA on one or other of the q' or p' arm of the target chromosome;

selecting for cells expressing the selectable marker;

introducing into said cells a second truncation construct comprising the

other of said q' or p' targeting DNA;

selecting for cells expressing the selectable marker associated with said second truncation construct; and

then isolating the truncated chromosome which comprises a neocentromere.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a diagrammatic representation showing neocentromere-based mini-chromosomes (abbreviated as NC-MiC) formation from the mardel(10) chromosome. **(A)** Structure of TACT targeting constructs. Targeting DNA from the p' or q' arms of mardel(10) were cloned into vectors containing small arrays of cloned human telomeric DNA (Htel) adjacent to a mammalian selectable marker (hygromycin^R, neomycin^R, or puromycin^R). Constructs were linearized at a restriction site between the vector DNA and the telomere repeats to expose the telomere sequences at the terminal. Following a homologous recombination event, the vector DNA should not be incorporated into the host chromosome. Loss of vector DNA allowed design of a screening assay for possible recombination events. **(B)** Schematic formation of mardel(10) and NC-MiCs. Open arrows indicate the breakpoints on the normal chromosome 10 in the generation of mardel(10). The long and short arms of mardel(10) are denoted as q' and p', respectively. The truncation events resulting in the formation of NC-MiCs 1-5 are represented by Tc1 and Tc2. Location of the zeocin resistance gene inserted into mardel(10) is indicated by an asterisk. Position of BAC B10K1 used in a screening assay for loss of p' arm is shown. Bold lines above chromosome 10 denote the locations and designations of sub-chromosomal DNA paints used to characterize the NC-MiCs. **(C)** Mapping of the NC-MiCs. Ordered cosmid and BAC clones covering approximately 3 Mb and previously mapped to the 10q25 neocentromere region are shown. Vertical shaded area represents the E8 BAC containing the centromere protein-binding neocentromere (NC) domain (23,27). Open arrowheads to the left and right of E8 indicate intended positions of targeted truncation using q' and p' truncation constructs. (+) denotes a positive FISH result for a BAC or cosmid probe on an NC-MiC, while (–) indicates a negative FISH result. The approximate sizes of the different NC-MiCs are shown in parentheses. **(D)** Characterization of the targeted q' truncation site. The targeting DNA for q' truncation is a 6-kb *Xba*I fragment subcloned from cosmid Y3C94 (also present in BAC B79E16) and is represented by the hatched box. Locations of PCR probes from B79E16 are indicated by double-headed arrows and are denoted F1, F2, and F3. Hybridization status of these probes

on NC-MiCs is denoted by (+) or (–) confirming that the q' truncation was the result of a targeted event.

Figure 2 is a photographic representation of FISH and/or immunofluorescence analysis of ZB30 and NC-MiC2. **(A)** FISH analysis of ZB30 showing hybridization of E8 (green) and tagging of mardel(10) (arrowhead) with zeocin resistance gene (red). **(B-H)** FISH and/or immunofluorescence analysis on NC-MiC2. Normal chromosome 10 is indicated by arrowhead and NC-MiC2 by arrow. (i-iv) Combined image, and split images for green, red, and DAPI, respectively. **(B)** FISH using E8 (green) and Y3C94 cosmid probe (red), showing presence of Y3C94 on NC-MiC2. **(C)** FISH using E8 (green) and q' cosmid Y3C109 (red), showing absence of Y3C109 on NC-MiC2. **(D)** FISH using E8 (green) and a PCR fragment F1 (red) derived from Y3C94/B79E16 and overlapping the q' targeting DNA (see Figure 1D), showing presence of F1 DNA on NC-MiC2. **(E)** FISH using E8 (green) and a PCR fragment F2 (red) derived from B79E16 in a region immediately distal to the targeting DNA (see Figure 1D), showing absence of F2 DNA on NC-MiC2. **(F)** FISH using E8 (green) and immunofluorescence using anti-CENP-B antibody (red), showing absence of CENP-B on NC-MiC2. **(G)** FISH using E8 (green) and immunofluorescence using anti-CENP-E antibody (red), showing presence of CENP-E protein on NC-MiC2. **(H)** FISH using E8 (green) and telomere-repeat PNA probe (red), showing telomeric sequences on NC-MiC2.

Figure 3 is a photographic representation of FISH and/or immunofluorescence analysis of NC-MiC3. Normal chromosome 10 is indicated by arrowhead and NC-MiC3 by arrow. (i-iv) Combined image, and split images for green, red, and DAPI, respectively. **(A)** FISH using E8 (green) and q' BAC B79E16 (red), showing presence of B79E16 on NC-MiC3. **(B)** FISH using E8 (green) and q' cosmid Y3C109 (red), showing absence of Y3C109 on NC-MiC3. **(C)** FISH using E8 (green) and p' cosmid Y13C12 (red), showing presence of Y13C12 on NC-MiC3. **(D)** FISH using E8 (green) and p' BAC B179N3 (red), showing absence of B179N3 on NC-MiC3. **(E)** FISH using E8 (green) and a pan-α-satellite pTRA-7 probe (red), showing absence of α-satellite on NC-MiC3. **(F)** FISH using E8 (green) and immunofluorescence using anti-CENP-E antibody (red), showing presence of CENP-E on

NC-MiC3. **(G)** FISH using E8 (green) and immunofluorescence using anti-CENP-F antibody (red), showing presence of CENP-F on NC-MiC3.

Figure 4 is a photographic representation of FISH and/or immunofluorescence analysis of NC-MiC4. Normal chromosome 10 is indicated by arrowhead and NC-MiC4 by arrow. (i-iv) Combined image, and split images for green, red, and DAPI, respectively. **(A)** FISH using E8 (green) and p' cosmid Y13C12 (red), showing the presence of Y13C12 on NC-MiC4. **(B)** FISH using E8 (green) and p' BAC B43A11 (red), showing the absence of B43A11 on NC-MiC4. **(C)** FISH using E8 (green) and pan-α-satellite pTRA-7 probe (red), showing absence of α-satellite on NC-MiC4. **(D)** FISH using TTAGGG telomere-repeat PNA probe, showing positive signals on all human telomeres but not on NC-MiC4. **(E)** FISH using E8 (green) and immunofluorescence using anti-CENP-B antibody (red), showing absence of CENP-B on NC-MiC4. **(F)** FISH using E8 (green) and immunofluorescence using anti-CENP-C antibody (red), showing presence of CENP-C on NC-MiC4. **(G)** FISH using E8 (green) and immunofluorescence using anti-CENP-E antibody (red), showing presence of CENP-E on NC-MiC4. **(H)** FISH using E8 (green) and immunofluorescence using anti-CENP-F antibody (red), showing presence of CENP-F on NC-MiC4.

Figure 5 is a photographic representation of FISH and/or immunofluorescence analysis of NC-MiC5. Normal chromosome 10 is indicated by arrowhead and NC-MiC5 by arrow. (i-iv) Combined image, and split images for green, red, and DAPI, respectively. **(A)** FISH using E8 (green) and p' BAC BA48L24 (red), showing presence of BA48L24 on NC-MiC5. **(B)** FISH using E8 (green) and p' BAC BA69K10 (red), showing absence of BA69K10 on NC-MiC5. **(C)** FISH using E8 (green) and pan-α-satellite pTRA-7 probe (red), showing absence of α-satellite on NC-MiC5. **(D)** FISH using E8 (green) and immunofluorescence using anti-CENP-B antibody (red), showing absence of CENP-B on NC-MiC5. **(E)** FISH using E8 (green) and immunofluorescence using anti-CENP-A antibody (red), showing presence of CENP-A on NC-MiC5. **(F)** FISH using E8 (green) and immunofluorescence using anti-CENP-C antibody (red), showing presence of CENP-

C on NC-MiC5. **(G)** FISH using E8 (green) and immunofluorescence using anti-CENP-E antibody (red), showing presence of CENP-E on NC-MiC5.

Figure 6 is a photographic representation of chromosome-painting analysis of NC-MiCs. **(A)** Whole-chromosome paints for all 24 human chromosomes (1-22, X, Y). Left panel shows positive painting (green) on normal human chromosomes, except for the pericentric heterochromatic regions and those on the short arms of acrocentric chromosomes, and the q12 region of Y. Upper right panel shows the corresponding painting result for NC-MiC4 while lower right panel shows the result for NC-MiC5. All chromosome paints with the exception of chromosome 10 are negative on the NC-MiCs as evidenced by a lack of overlapping paint (green) and E8 (red) signals (confirmed by splitting individual images; not shown), while the chromosome-10 paint signal is clearly seen on NC-MiCs as evidenced by an overlap of red and green signals producing a combined yellow colour. **(B)** Subchromosome-10 paints on NC-MiC5. Positions for these subchromosome paints on chromosome 10 are shown schematically in Figure 1B. Left-hand panels show designations of the paints and FISH results (green) on normal chromosome 10. Right-hand panels show the negative results obtained for these paints on NC-MiC5. E8 signals are in red. Chromosomes and NC-MiCs are not shown to scale.

Figures 7A-C are diagrammatic representations showing NC-MiC6 construction *via* the truncation of NC-MiC2. **(A)** Structure of TACT targeting constructs used for truncating the p' arm from NC-MiC2. Targeting DNA from the p' arm of mardel(10) and a mammalian selectable marker (blasticidin resistance gene, blasticidin^R) were cloned into vectors containing small arrays of cloned human telomeric DNA (Htel) adjacent to an antibiotic resistance gene, neomycin^R. Constructs were linearized at a restriction site between the vector DNA and the telomere repeats to expose the telomere sequences at the terminal. Following a homologous recombination event, the blasticidin resistance gene should not be incorporated into the host chromosome. Loss of the blasticidin resistance gene allowed the design of a screening assay for possible recombination events. **(B)** Schematic formation of mardel(10) and NC-MiCs. Open arrows indicate the breakpoints on the normal chromosome 10 in the generation of mardel(10). The long and short arms of mardel(10)

are denoted as q' and p', respectively. NC-MiC2 was truncated at B79e16/Y3C94 (Tc1) (as described earlier) from mardel(10), whereas NC-MiC6 is a result of truncation 2, Tc2, at B137iL/Y13c15 using the TACT construct described in (A). **(C)** Mapping of the NC-MiCs 2 and 6. Ordered cosmid and BAC clones covering approximately 3 Mb and previously mapped to the 10q25 neocentromere region are shown. the vertical shaded area represents the centromere protein CENP-A-binding domain (51). Open arrowheads indicate positions of targeted truncation. (+) denotes a positive FISH result for a BAC or cosmid probe on an NC-MiC, while (-) indicates a negative FISH result. The approximate sizes of the different NC-MiCs are shown in parentheses.

Figure 8 is a tabular and graphical representation showing the results of culturing NC-MiC6 for 60 divisions either in the presence or absence of neomycin drug G418 at a concentration of 150 μg/ml before they were harvested at various intervals for determination of stability. The X axis represents the number of divisions, whereas the Y axis represents the percentage of NC-MiC present in culture as determined by FISH analysis. The results indicated mitotic stability of NC-MiC-6 in the presence or absence of G418.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is predicated in part on the identification and isolation of nucleic acid molecules exhibiting neocentromeric properties. In accordance with the present invention, a neocentromere is considered a centromere which does not contain substantial amounts of repetitive DNA sequences that are present on a normal centromere (e.g. α-satellite in humans and minor satellite in mouse) and, when activated, is capable of functioning as a centromere. For example, a mammalian (e.g. human) neocentromere is a centromere which does not contain substantial α-satellite DNA repeat sequences. The term "substantial" in this context means that the nucleic acid molecule does not contain detectable normal centromeric repetitive DNA sequences such as α-satellite by FISH analysis under medium stringency conditions or by direct sequence comparison under medium homology criteria. The neocentromere may, however, contain a small number of highly diverged normal centromeric repetitive DNA sequences. In primates, for example, α-satellite DNA is considered to be about 170 bp in length. An nucleic acid molecule containing an activated neocentromere or a neocentromere otherwise functioning as a centromere facilitates in accordance with the present invention, the nucleic acid molecule in the form of a mini-chromosome or pre-fabricated with other DNA to facilitate transfection, replicating, remaining extra-chromosomal and segregating with cell division. Reference herein to "neocentromere" is taken to mean a centromere substantially devoid of repetitive DNA sequences that are normally present on the centromere of an endogenous and structurally unaltered chromosome such as α-satellite DNA repeat sequences on human chromosomes. Furthermore, a neocentromere is considered to be derived from a normally non-centromeric region of the genome.

Accordingly, one aspect of the present invention provides an isolated nucleic acid molecule comprising a sequence of nucleotides which defines an eukaryotic neocentromere.

More particularly, the present invention provides an isolated nucleic acid molecule comprising a sequence of nucleotides derived from a eukaryotic chromosome and

encompassing a neocentromere which nucleic acid molecule exists in a truncated or modified form in a compatible cell or, when introduced by transfection as a pre-fabricated DNA entity into a compatible cell, is capable of replicating, acting as an extra chromosomal element and segregating with cell division.

The present invention is exemplified herein by the identification of a human neocentromere. This is done, however, with the understanding that the present invention extends to all eukaryotic neocentromeres such as from mammalian, plant, avian, insect, worm, fungal, yeasts and reptilian chromosomes. The most preferred neocentromere, however, is from human chromosomes and their mammalian homologs.

The present invention is predicated in part on the construction of a number of neocentromere-based human mini-chromosomes using a combination of targeted telomere-associated truncation of the q' arm and apparently random truncation of the p' arm of the mardel(10) chromosome. The latter refers to a chromosome identified in a human patient and results from a re-arrangement of human chromosome 10. The mardel(10) marker is mitotically stable and, in accordance with the present invention, contains a functional neocentromere at a location regarded as non-centromeric. The neocentromere at mardel(10) is located between q24 and q26 on chromosome 10 and more particularly around q25. Even more particularly, the neocentromere maps to q25.2 on chromosome 10. The present invention is exemplified by DNA cloned from the q24-q26 region of the mardel(10) chromosome as well as the corresponding region on normal human chromosome 10. These DNA molecules contain a functional neocentromere. The present invention extends, however, to any neocentromere on any chromosome in mammalian and non-mammalian animals as well as plants, insects, worms, fungal, reptiles and yeasts.

Detailed mapping using cosmid and BAC probes from the 10q25 region allowed the truncation sites to be defined and the demonstration that the NC-MiCs contain single-copy intact DNA from this region. Extensive FISH using pan-α-satellite DNA, whole-chromosome paints for all human chromosomes and different sub-chromosomes-10 paints, revealed that none of the NC-MiCs have acquired detectable amounts of α-satellite DNA

or other human genomic sequences. Based on the mapping data, the sizes of NC-MiCs 3, 4 and 5, were estimated to be approximately 1.6, 1.6 and 0.8 Mb, respectively.

The present invention further contemplates a nucleic acid molecule or its chemical equivalent having a tertiary structure which defines a human neocentromere or a functional derivative thereof or a latent, synthetic or hybrid form thereof or its mammalian or non-mammalian homolog.

Even more particularly, the present invention is directed to an isolated nucleic acid molecule having a sequence of nucleotides or their chemical equivalents which directs a conformation defining a human neocentromere or a functional derivative thereof or a latent, synthetic or hybrid form thereof or its mammalian or non-mammalian homolog wherein the centromere associates with centromere binding proteins such as CENP-A and CENP-C but not limited to these proteins.

Reference herein to "latent" in relation to a centromere includes reference to a centromere not normally functional but nevertheless activatable under certain conditions. A latent centromere may also be considered as a neocentromere provided it has no substantial repetitive DNA sequences which are found on normal centromeres such as α-satellite DNA repeat sequences. A preferred repetitive DNA sequence in a non-human chromosome is considered to be a functionally equivalent repeat DNA to α-satellite DNA.

Accordingly, another aspect of the present invention contemplates the use of any method for identifying a neocentromere or a functional homolog, said method includes isolating DNA by chromatin immunoprecipitation using an antibody specific to mammalian CENP-A and/or CENP-C, or an antibody capable of cross interacting with mammalian CENP-A and/or CENP-C, amplifying the DNA isolated by immunoprecipitation and incorporating label into the amplified DNA and then using the amplified DNA to probe a DNA array comprising genomic DNA or its equivalent and identifying and isolating clones which hybridize to said immunoprecipitated DNA (52).

The size of the neocentromere in accordance with the present invention may range from about 50 bp to about 2000 kbp, from about 70 bp to about 1000 kbp, from about 75 bp to about 800 kpb, from about 80 bp to about 500 kpb, from about 85 bp to about 200 kpb, from about 90 bp to about 100 kbp, from about 100 bp to about 1 kpb, about 120 bp to about 500 bp, about 180 bp to about 300 bp. In one embodiment, the neocentromere is approximately 60-100 kbp. In another embodiment, the neocentromere is about 80 kpb. In a particularly preferred embodiment, the neocentromere is from about 50 kbp to about 2000 kpb. The neocentromere may encompass different structurally or functionally distinct domains such as CENP-A-binding or other centromere protein-binding domain, or domains showing different replication timing, chromatin structure, scaffold organisation, chemical modification status (e.g. acetylation, methylation, phosphorylation, poly-ADP-ribosylation). Provision is also given to the size of the neocentromere being larger than 2000 kbp to encompass all the functionally important domains.

The size of the mini-chromosome in accordance with the present invention may range from about 500 bp to about 20000 kbp, from about 700 bp to about 1000 kbp, from about 750 bp to about 8000 kpb, from about 800 bp to about 5000 kpb, from about 850 bp to about 2000 kpb, from about 900 bp to about 1000 kbp, from about 1000 bp to about 10 kpb, about 1200 bp to about 5000 bp, about 1800 bp to about 3000 bp. In one embodiment, the mini-chromosome is approximately 600-1000 kbp. In another embodiment, the mini-chromosome is about 800 kpb. In a particularly preferred embodiment, the neocentromere is from about 500 kbp to about 20000 kpb. The mini-chromosome may encompass different structurally or functionally distinct domains such as a neocentromere, replication origins, and telomeres. In a particularly preferred embodiment, the mini-chromosome contains a active neocentromere.

The nucleic acid molecule of the present invention may comprise a naturally occurring nucleotide sequence from a healthy human subject or may comprise the nucleotide sequence from a human subject exhibiting one or more chromosomal-dependent conditions such as a subject carrying mardel(10) chromosome or a chromosome conferring an equivalent or similar condition or may carry one or more nucleotide substitutions, deletions

and/or additions relative to the naturally or non-naturally occurring sequence. Such modifications are referred to herein as "derivatives" and include mutants, fragments, parts, homologs and analogs of the naturally occurring nucleotide sequence. Preferably, the derivatives of the present invention still define a functional neocentromere.

Reference herein to a "neocentromere" includes reference to a functional neocentromere or a functional derivative thereof meaning that it is capable of facilitating sister chromatid cohesion and chromosomal segregation during mitotic cell divisions and/or is capable of associating with CENP-A and/or CENP-C and/or other functionally important centromere proteins and/or is capable of interacting with anti-CENP-A antibodies or anti-CENP-C antibodies or antibodies to other functionally important centromere proteins. Generally, and preferably, the neocentromere is incapable of interacting with CENP-B or anti-CENP-B antibodies. Alternatively, the neocentromere may be a latent centromere capable of activation by epigenetic mechanisms or other relevant mechanisms. The neocentromere may also be a hybrid or other human, mammalian, plant or yeast neocentromeres. Synthetic neocentromeres provided by, for example, polymeric techniques to arrive at the correct conformation are also contemplated by the present invention. All such forms and definitions of neocentromeres are encompassed by use of this term.

Another aspect of the present invention provides an isolated nucleic acid molecule or chemical equivalent which comprises a nucleotide sequence or chemical equivalent directing a conformation which defines a neocentromere or a functional derivative thereof or a latent, synthetic or hybrid form thereof and wherein said neocentromere is substantially devoid of normal centromeric repetitive DNA such as α-satellite DNA and wherein the neocentromere is capable of associating with CENP-A or CENP-C or other functionally important centromere-binding proteins or antibodies thereto.

Preferably, the neocentromere is incapable of interacting with CENP-B or antibodies thereto.

In one embodiment, the neocentromere corresponds to a human genomic region which

maps between q24 and q26 on chromosome 10 and in particular q25 on chromosome 10.

The nucleic acid molecule or its chemical equivalent of the present invention defining a conformational neocentromere or functional derivative thereof or latent, synthetic or hybrid form thereof is useful *inter alia* for the generation of mini-chromosomes or artificial chromosomes such as human neocentromere-based mini-chromosomes (NC-MiCs), human artificial chromosomes (HACs), mammalian artificial chromosome (MACs), yeast artificial chromosomes (YACs) and plant artificial chromosomes (PLACs). Human NC-MiCs are particularly useful since they are capable of accommodating large amounts of DNA and are capable of propagation in human cells. The NC-MiCs are non-viral in origin and, hence, are more suitable for gene therapy by, for example, introducing therapeutic genes, than conventional viral based vector systems. Furthermore, the NC-MiCs remain extra-chromosomal and, hence, have no insertional/substitutional mutagenic potential. The essence of a NC-MiCs is the presence of a neocentromere or latent, synthetic or hybrid form thereof which enables stable segregation during cell division. The NC-MiCs also remain extra-chromosomal and, hence, are more suitable for gene therapy. Reference to "extra-chromosomal" means that it does not integrate into the main chromosome and, in effect, is episomal.

Accordingly, the present invention provides a genetic construct comprising an origin of replication for a eukaryotic cell and a nucleic acid molecule encompassing a eukaryotic neocentromere or a functional derivative thereof or a latent, synthetic, hybrid form thereof or its mammalian or non-mammalian homolog flanked by telomeric nucleotide sequences functional in the cell in which the genetic construct is to replicate and where said genetic construct when introduced into a cell is a replicating, extra chromosome element, either in a circular or linear form, which segregates with cell division.

More particularly, the present invention further contemplates a genetic construct in the form of a mini-chromosome or an artificial chromosome comprising an origin of replication for a mammalian, human, plant or yeast cell and a nucleic acid molecule encompassing a human neocentromere or a functional derivative thereof or a latent,

synthetic or hybrid form thereof or its mammalian or non-mammalian homolog flanked by telomeric nucleotide sequences or in a circular form carrying minimal or no telomere sequences functional in the cell in which the mini-chromosome or artificial chromosome is to replicate.

Another embodiment provides a genetic construct in the form of a mini-chromosome or an artificial chromosome comprising an origin of replication for a mammalian, human, plant or yeast cell and a nucleic acid molecule having a tertiary structure which defines a human neocentromere or a functional derivative thereof or a latent, synthetic or hybrid form thereof or its mammalian homolog flanked by telomeric sequences or in a circular form carrying minimal or no telomere sequences functional in the cell in which the mini-chromosome or artificial chromosome is to replicate.

Yet another embodiment is directed to a genetic construct in the form of a mini-chromosome or an artificial chromosome comprising an origin of replication for a mammalian, human, plant or yeast cell and a nucleic acid molecule having a sequence of nucleotides which directs a conformation defining a human neocentromere where the centromere associates with CENP-A and/or CENP-C and/or other centromere proteins or antibodies thereto and does not contain substantial α-satellite DNA repeat sequences, said nucleic acid molecule flanked by telomeric nucleotide sequences or in a circular form carrying minimal or no telomere sequences functional in the cell which the mini-chromosome or artificial chromosome is to replicate.

The present invention further provides a method for generating a mini-chromosome, said method comprising:-

introducing into a cell a truncation construct comprising a vector having telomeric sequences, a selectable marker, homologous targeting DNA on one or other of a q' or p' arm of the target chromosome;

selecting for cells expressing the selectable marker;

introducing into said cells a second truncation construct comprising the other of said q' or p' targeting DNA;

selecting for cells expressing the selectable marker associated with said second truncation construct; and

then isolating the truncated chromosome which comprises a neocentromere.

In one embodiment, the q' and p' targeting arms target the mardel(10) chromosome.

In a particularly preferred embodiment, the mini-chromosome is defined as NC-MiC1-5 as defined in Figure 1.

The present invention further provides a cell comprising a chromosome having q' and p' arms flanking a neocentromere wherein one or both q' and p' arms are truncated.

The above described method or its modified version thereof may also be used to introduce a gene or other nucleotide sequence such as for expression.

The present invention extends to eukaryotic cells such as human, primate, insect, yeast or other eukaryotic cells carrying the genetic constructs of the present invention and to proteins produced therefrom.

The genetic constructs of the present invention include mini-chromosomes and artificial chromosomes as well as DNA constructs useful in the generation of mini-chromosomes and artificial chromosomes.

The genetic constructs may also comprise marker genes, unique restriction sites, or recombination enhancing marker (e.g. LoxP DNA, for use with Cre recombinase) to facilitate insertion of adventitious DNA. Accordingly, the genetic constructs of the present

invention may further comprise adventitious or heterologous DNA encoding a product of interest. Preferred products of interest include pharmaceutically useful genes such as genes encoding cytokines, receptors, growth regulators and the like. Endogenous genes may also be replaced by wild-type genes or modified genes.

Specific DNA sequences intrinsically present on the genetic constructs (e.g. putative ESTs or expressed genes or unstable DNA elements) may also be identified, removed or modified by further genetic manipulation or engineering. Other DNA components (e.g. therapeutic or marker genes, LoxP DNA sequences, unique restriction sites such as I-SceI) may also be subsequently added to the genetic constructs to improve or broaden their utility or application.

The adventitious or heterologous DNA may also encode a molecule not synthesized in a sufficient amount in a particular subject and hence the increased copy number permits greater amounts of the molecule being synthesized.

Accordingly, the present invention contemplates a genetic construct comprising an origin of replication and a first nucleic acid molecule defining a human neocentromere or a functional derivative thereof or latent, synthetic or hybrid form thereof or a mammalian or non-mammalian homolog, a second nucleic acid molecule encoding a peptide, polypeptide or protein, wherein said first and second nucleic acid molecules are flanked by human telomeric sequences or in a circular form carrying minimal or no telomeric sequences functional in the cell in which the genetic construct is to replicate.

Reference herein to "segregate" preferably means mitotically stable segregation. Conveniently, stable segregation may be determined as the presence of a mini-chromosome or an artificial chromosome in more than 40-60% of daughter cells after 4-6 months of continuous passage.

The present invention extends to other mini-chromosomes or artificial chromosome analogs to the NC-MiCs described above such as HACs, MACs and PLACs, or similar

entities by any other descriptions or names such as micro-chromosomes, synthetic chromosomes, and variations thereof.

Another aspect of the present invention relates to peptides, polypeptides and proteins which bind, interact or otherwise associate with the human neocentromere of the present invention or its mammalian and non-mammalian homolog. Preferably, the molecules are proteins, referred to as primary (1°) proteins. The 1° proteins bind to the neocentromere and secondary (2°) proteins bind to the 1° proteins before or after association with the neocentromere. The identification of the human neocentromere in accordance with the present invention provides a mechanism for assaying 1° proteins and 2° proteins which may be important for screening chromosomes in, for example, genetic disorders. This is particularly the use in Down's Syndrome which results from defective chromosome segregation.

The 1° proteins are readily detected by, for example, a gel shift assay. The nucleic acid molecule of the present invention defining the human neocentromere is digested, labelled and contacted with nuclear extract putatively containing the 1° proteins and resolved on a gel. When a 1° protein binds to a fragment carrying a binding portion of the neocentromere, the DNA fragment migrates in the gel at a slower rate due to the bound protein.

The present invention extends to purified 1° proteins capable of association with the subject centromere and to genetic sequences encoding same and to antibodies thereto.

The neocentromeres of the present invention are readily identified and characterized using, for example, human fibrosarcoma cell lines. For example, DNA suspect of carrying a neocentromere is introduced into fibrosarcoma cells in a linear form generally together with a telomeric sequence. The cells are then screened for the presence of replicating, extra chromosomal and segregating elements, referred to as artificial chromosomes.

The present invention further encompasses eukaryotic cells carrying replicating, extra-

chromosomal and segregation nucleic acid molecules. Preferably, the eukaryotic cells are mammalian cells and most preferably human cells. The nucleic acid molecules according to this aspect of the present invention are preferably as herein described.

The following cell lines were deposited at the ECACC, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wilts, SP4 0JG, United Kingdom on 20 December 2000, under the listed Accession Numbers:-

Cell Line	Acronyn	Accession Number
CHO/BE ZB30	-	00122001
HT1080 MIC 1	NC-MiC1	00122002
HT1080-MIC 2	NC-MiC2	00122003
HT1080-MIC 3	NC-MiC3	00122004
HT1080-MIC 4	NC-MiC4	00122005
HT1080-MIC 5	NC-MiC5	00122006
HT1080-MIC 5a	NC-MiC5a	00122007
HT1080-MIC 5b	NC-MiC5b	00122008

The present invention is further described by the following non-liming Examples.

EXAMPLE 1

Cell culture and chemicals

BE2Cl-18-5f (abbreviated 5f) was cultured as previously described (27). HT1080 and derivatives were cultured in DMEM (Gibco BRL) with 10% v/v fetal calf serum (FCS). Hygromycin (Roche), Puromycin (Sigma corp), or Zeocin (Invitrogen) were added to medium at concentrations of 250 μg/ml, 1 μg/ml, or 200 μg/ml, respectively. Medium was supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco BRL). All cell lines were maintained at subconfluence. Microtubule depolymerizing agents colcemid (Gibco BRL) or nocadazole (Sigma) were added to medium at concentrations of 10 μM or 0.1 μg/ml for 1 or 6-12 hours, respectively, prior to cell harvesting. All chemicals used were of molecular biology grade and purchased from commercial sources.

EXAMPLE 2

Transfection of cell lines

Transfection of 5f and ZB30 cell lines were carried out using electroporation. Briefly, 10^7 log-phase cells were harvested, washed twice in PBS and resuspended in 800 μl electroporation buffer (20 mM Hepes, pH 7.0, 150 mM NaCl, 5mM KCl, 6 mM glucose, 2.5 mM NaOH, 1 mM Na_2HPO_4). 20 μg of linearized DNA was mixed with cells in a 0.4 cm cuvette and cells were electroporated at 1.2kV, 25uF using a Biorad Gene Pulser Electroporator. Transfection of HT1080 or derivative cells was carried out using either electroporation as previously described (30) or lipofection. For lipofection, the cells were plated at 1-3 x 10^5 cells/ml in 40 ml on a 150 mm plate one day prior to transfection. This resulted in 50-70% confluency on the day of the experiment. 2 ml of serum-free DMEM was used to dilute 100 μl Fugene 6 transfection reagent (Boehringer Mannheim) and the suspension was incubated at 37°C for 5 min. 20 μg of DNA was also diluted in 2 ml of serum-free DMEM. The diluted Fugene 6 was then added onto the DNA suspension drop-wise. The mixture was gently tapped and incubated for 15 min at room temperature. Following incubation, the DNA/Fugene 6 mixture was added onto the cells drop-wise. Cells were seeded into 96-well plates and selection was applied 24-48 hours post

transfection.

EXAMPLE 3

Microcell mediated chromosome transfer to HT1080

Microcell fusion was carried out as previously described (38). Log-phase donor ZB30 cells were incubated with colcemid (1 μg/ml) overnight. Cells were harvested after 48 hours and resuspended in percoll/serum-free DMEM (1:1) supplemented with 20 μg/ml of cytochalasin B (Sigma). The cell suspension was then subjected to centrifugation at 18,000 rpm for 90 min at 32°C in oakridge tubes (Nalgene). Both bands of cell-mix were pelleted and washed once with serum-free DMEM prior to filtration through isopore membranes of 30, 8 and 5 μM (Millipore Corp., MA). The microcells were then resuspended in serum-free DMEM containing 10 μg/ml PHAP (Difco, West Molesey, UK), and were allowed to agglutinate with the recipient HT1080 cells for 45min at 37°C. Following agglutination, cells were fused by addition of 50% w/v PEG (Boehringer Mannheim) and incubated for 2 min at room temperature followed by rinsing with serum-free DMEM. After incubation, cells were cultured overnight in DMEM containing 10% v/v FCS. The medium was then replaced with DMEM containing 200 μg/ml of zeocin and cells were maintained in selection for a period of 14 days before colonies were picked for further characterization.

EXAMPLE 4

FISH/Immunofluorescence

Combined FISH/Immunofluorescence was carried out using a modified procedure previously described (25,51). FISH using pan-α-satellite probe pTRA7 and PNA-FISH of telomeric sequences (Perspectives Biosystem, MA) were carried out as previously described (39,40). Epifluorescence microscopy was performed on a Zeiss Axoplan II (Carl Zeiss, Carnegie, Australia) mounted with appropriate filter sets. Images were digitally acquired using a cooled charged-coupled device video camera (SenSys 2, Photometrics, Tuscon, AZ, USA) connected to a PowerMac G3 personal computer controlled by the software IP Lab Version 2.5.5 (Scanalytics Inc., Fairfax, VA,USA). Chromosome painting

experiments were carried out using WCP Chromosome Paint Kit (Vysis Inc.) according to the manufacturer's instructions. Subchromosome-10 paints were derived from somatic cell radiation hybrid genomic DNA obtained from M. Rocchi (University of Bari). Inter-Alu amplification of somatic cell hybrid DNA was carried out using primers 5' GGATTACAGGYRTGAGCCA [SEQ ID NO:1] and 5' RCCAYTGCACTCCAGCCTG [SEQ ID NO:2] as previously described (41). Labelling of paint-probes and FISH were carried out using standard techniques.

Polyclonal anti-CENP-A, monoclonal anti-CENP-B, polyclonal anti-CENP-C, and CREST-6 antisera have been previously described (42,43,44,27). Polyclonal anti-CENP-E (45), anti-CENP-F (46), and anti-hBUB1 (47) were provided by T.J. Yen (Fox Chase Cancer Center), polyclonal anti-hZW10 (48) by B. Williams and M.L Goldberg (Cornell University), polyclonal p55CDC (49) by J. Weinstein (Amgen Corp) and polyclonal anti-TRF1 (32) by Titia de Lange (Rockefeller University, NY). Antisera CREST-6 was from a patient with the autoimmune CREST disease, containing antibodies against centromere components including CENP-A and CENP-B (51). Other secondary antibodies were purchased from the Jackson ImmunoResearch Laboratory Inc. (West Grove, Pennsylvania, USA).

EXAMPLE 5

Truncation constructs

Truncation constructs contained either pGK:hygromycin, pGK:puromycin, or pGK:neomycin resistance gene cassettes. A 2kb array of human telomeric repeats was obtained from pBS Sal-tel(5) plasmid (28,50). Genomic cosmid clones containing DNA corresponding to the p' and q' arms of mardel(10) have been previously described. 5-10 kb fragments lacking high-copy repeat DNA sequences (as evidenced by a lack of COT-1 hybridization following Southern hybridization), were subcloned in both orientations into truncation vectors using standard techniques. Alternatively genomic DNA was PCR amplified directly from BAC clones using Long Range PCR kit (Boehringer Mannheim) for cloning into truncation vectors. All truncation constructs were made in pAlter

(Promega Corp.) vector backbone.

EXAMPLE 6

Concatamerization of zeocin resistance marker

A zeocin resistance cassette from the pZeoSV2(+) plasmid (Invitrogen) was PCR amplified with the addition of flanking *Not*I restriction sites. This was cloned into pGEM-T (Promega Corp.). 100 µg of this plasmid was digested with *Not*I, and purified by phenol/chloroform extraction. To produce concatamers, 10 µl ligase buffer, 40 units of T4 ligase, and 5 µl of 100mM ATP were added to the digested DNA in a total volume of 100 µl. The ligation reaction was carried out overnight and 1 µl of the completed reaction was tested by pulsed field gel electrophoresis to determine the size of the resulting concatamers. DNA was phenol/chloroform-extracted and ethanol-precipitated prior to transfection.

EXAMPLE 7

p' arm truncation screening using dot blot hybridization

Cells in 24-well plates were harvested by trypsinization and transferred directly to a Minifold dot blotting apparatus (96 per blot; Schleriche and Schuell Inc.) containing Hybond N+ (Amersham Corp.) under vacuum. Following transfer, Hybond N+ was denatured for 10 min (1M NaOH, 1M NaCl), neutralized twice for 5 min (1 M Tris-HCl, 1.5 M NaCl) followed by a final wash in 2X SSC. Membranes were then baked for 1.5 hours at 80°C and were probed using ^{32}P-labelled pAlter vector (Promega Corp.) DNA using standard techniques.

EXAMPLE 8

Mardel(10) tagging and transfer into HT1080 cells

A CHO-based somatic cell hybrid line (designated 5f) containing the mardel(10) chromosome (27) was used. Initial telomere-associated chromosome truncation (TACT)

using a system similar to that previously described (9,12,28) was performed on 5f employing the truncation vectors shown in Figure 1A (described below). An exhaustive screening of over 25,000 drug-resistant colonies transfected with different truncation vectors yielded no positive truncation events, suggesting that the 5f cell line was not a suitable host for TACT. The inventors then decided to transfer the mardel(10) chromosome into human HT1080 cells since this cell line is known to be homologous recombination proficient (29,30), shows telomerase activity (31,32,33), and is a good recipient for microcell-mediated chromosome transfer (34,35). A random insertion approach was first used to tag mardel(10) in 5f cells with a zeocin resistance gene cloned in pGEM-T vector. In order to facilitate subsequent FISH screening for chromosome tagging, the zeo^R/pGEM-T construct containing zeocin resistance gene was concatamerized and only DNA fragments larger than 50 kb were used to transfect 5f cells. Screening 63 individual zeocin-resistant colonies identified a single cell line (designated ZB30) in which mardel(10) was tagged at its distal q' region (Figure 2A). This cell line was used as a donor in microcell-mediated chromosome transfer into HT1080 cells. 15 out of 60 zeocin-resistant microcell-fusion colonies were shown to contain mardel(10). Extensive FISH and immunofluorescence analyses of these cell lines using BACs from the neocentromere region, pan-α-satellite, CHO genomic DNA, and a host of anti-centromere antibodies confirmed that the mardel(10) chromosome and its neocentromere were intact, and that no integration of any α-satellite repeats or CHO DNA had occurred on the chromosome. One of these cell lines, designated ZBHT-14, was employed in subsequent truncation experiments.

In addition to cell lines such as ZBHT-14 which have acquired an intact mardel(10), a number of lines containing randomly truncated derivatives of this chromosome were also detected in the fusion products; the generation and transfer of such broken chromosomal fragments appeared to be a relatively common occurrence associated with the microcell fusion procedure (36). One cell line (designated NC-MiC1) was of interest because it carried a mini-chromosome that was shown by FISH to contain approximately 2 Mb of DNA around the previously mapped 10q25 neocentromere domain (27) (Figure 1C). This cell line was retained for further characterization and truncation studies.

EXAMPLE 9

Truncation constructs

A complete physical map was prepared containing over 50 BAC and cosmid clones covering approximately 3 Mb of the 10q25 neocentromere region (Figure 1C). Based on this map, a number of truncation constructs containing different targeting DNA from this region were designed. These constructs contained either hygromycin (for q'-arm truncation), neomycin or puromycin (for p'-arm truncation) resistance genes, terminal cloned human telomere sequences, and 5-10 kb of targeting DNA from either the q' or p' arm of mardel(10) (Figure 1A). Initial TACT experiments were performed on the q' arm, followed by truncation of the p' arm (Figure 1B).

EXAMPLE 10

Truncation of q' arm

ZBHT-14 and NC-MiC1 cell lines were used in q' truncation experiments. Both cell lines were transfected with a hyg^R (hygromycin resistance) truncation-construct containing a 6-kb targeting DNA derived from the Y3C94 cosmid (which overlaps with BAC B79E16; Figure 1C). This targeting site is situated approximately 0.2 Mb from the anti-centromere antibody binding NC domain (27).

For the ZBHT-14 cell line, initial screening for q' truncation of mardel(10) was performed by plating the resulting hygromycin-resistant colonies in zeocin. This allowed the identification of cell lines which have lost the zeocin-containing chromosome portion of mardel(10) and were, therefore, zeocin sensitive. From a total of 7,300 hygromycin-resistant colonies, 210 were shown to be zeocin sensitive. FISH analysis revealed that most of these colonies contained random truncations or other unknown rearrangements. One cell line (designated NC-MiC2) appeared to have undergone the desired targeted truncation and was characterized further.

FISH analysis of NC-MiC2 using a host of known cosmid or BAC clones mapped along the 10q25 neocentromere region revealed that clones on or proximal to the targeting DNA were present (e.g. Y3C94; Figure 2B), whereas all clones distal to the targeting DNA were absent (e.g. Y3C109; Figure 2C) (summarized in Figure 1C). To more closely determine the site of truncation, three ~10-kb PCR fragments (F1-F3) immediately adjacent to either side of the intended targeting site (Figure 1D) were prepared and used in FISH analysis. The results indicated that only the fragment (F1) located proximally to the targeting DNA gave a positive signal (Figure 2D), whilst the two distal fragments F2 and F3 were both negative (Figure 2E). Further FISH using TTAGGG telomere-repeat peptide nucleic acid (PNA) probe demonstrated seeding of telomeric sequences on the truncated chromosome (Figure 2H). Low-stringency FISH using pan-α-satellite probe and immunofluorescence using anti-CENP-B antibody (Figure 2F) confirmed that no α-satellite DNA has been inserted into the truncated chromosome. These data strongly support a targeted-truncation event leading to the removal of most of the q' arm of mardel(10) in the production of NC-MiC2.

Mitotic stability of NC-MiC2 was determined by comparing cells grown in the presence or absence of hygromycin over 20 cell divisions. A similar retention rate of 85% was observed for cells grown with or without drug selection, suggesting that NC-MiC2 was mitotically stable. To further investigate whether this chromosome maintained a functional neocentromere, immunofluorescence studies were carried out using CREST-6 autoimmune serum (27) and specific antibodies to the histone H3-like protein CENP-A, CENP-C, and the kinesin-like motor protein, CENP-E. Strong antisera signals that co-localized with the E8 BAC previously mapped to the neocentromere (NC) domain (37) were observed for all the proteins tested (e.g. Figure 2G) thereby demonstrating neocentromere function on NC-MiC2.

Transfection of the NC-MiC1 cell line with the Y3C94 DNA-containing q' truncation construct resulted in over 1000 hygromycin-resistant colonies. These were screened for the loss of the vector DNA contained in the construct (see Figure 1A and Example 7) that was indicative of a targeted truncation event. Detailed FISH analysis of one resulting cell line,

NC-MiC3 with q' cosmids and BACs, and the PCR probes F1-F3, demonstrated a correctly targeted truncation at the intended Y3C94 site (examples of FISH results are shown in Figures 3A-B, and summarized in Figure 1C). Further p' mapping and analysis of NC-MiC3 are described below.

EXAMPLE 11

Truncation of p' arm

NC-MiC2 was subjected to further truncation using constructs carrying puromycin or neomycin markers and targeting DNA from 3 different p' regions (Figure 1C). Puromycin- or neomycin-resistant colonies, generated through several independent transfection experiments, were screened for possible targeting by probing for loss of vector DNA as described above, as well as by dual-color FISH using E8 BAC and a distal p'-arm BAC (B10K1) (see Figure 1B) to identify truncated NC-MiC2 derivatives. This analysis indicated that most colonies did not appear to contain the intended targeted chromosomal truncations of NC-MiC2. However, two cell lines (NC-MiC4 and NC-MiC5) were identified that showed truncation at p' sites relatively close to the neocentromere antigen-binding region (27). These were characterized in greater detail.

EXAMPLE 12

Structure of NC-MiCs 3, 4 and 5

Figure 1C summarizes the results for detailed FISH mapping of NC-MiCs 3, 4 and 5 using probes of known map position (some examples are shown in Figures 3-5). All three NC-MiCs showed the expected q' truncation within Y3C94. On the p' arm, chromosome truncation was seen between BACs Y13C12(present)/B179N3(absent), Y13C12 (present)/B43A11(absent), and BA48L24(present)/BA69K10(absent), for NC-MiC3 (and its predecessor NC-MiC1), NC-MiC4, and NC-MiC5, respectively (Figures 3C/D, 4A/B, and 5A/B). The intensity of the positive FISH signals on the different NC-MiCs were indistinguishable from those seen on the normal chromosomes 10 in HT1080 cells for all the cosmid and BAC probes tested, suggesting that no duplication of DNA has occurred

during formation of the NC-MiCs. Low-stringency FISH hybridization using pan-α-satellite DNA probe (Figures 3E, 4C and 5C), and immunofluorescene using anti-CENP-B antibody (Figures 4E and 5D) demonstrated the absence of centromeric α-satellite DNA. When the different NC-MiC cell lines were analyzed by FISH using whole-chromosome paints for all 24 human chromosomes, only the chromosome 10-paint produced positive signals on the NC-MiCs, suggesting that no detectable amount of genomic DNA from other chromosomes have been translocated onto the NC-MiCs during their formation (Figure 6A). Further analysis using a number of subchromosome 10-paints (see Figure 1B for locations) similarly demonstrated the absence of DNA from non-10q25 regions on the NC-MiCs (Figure 6B). It can, therefore, be concluded that NC-MiCs 3, 4 and 5 each contain single-copy DNA derived solely from the 10q25 neocentromere region, with total sizes estimated of approximately 1.6, 1.6 and 0.8 Mb, respectively (Figure 1C).

High-molecular weight genomic DNA was prepared from the different cell lines and subjected to pulsed field gel electrophoresis under varying conditions that resolved DNA of up to 6 Mb. Only the NC-MiC3 migrated into the gel, suggesting that this is a linear mini-chromosome and raising the possibility that the other NC-MiCs are circular structures. Comparison with yeast chromosome markers indicated a size of 1.6 Mb on the PFGE gel for NC-MiC3. FISH using a pan-telomere probe or immunofluorescence using an antibody to the telomere repeat-binding factor TRF1 produced signals on telomeric ends of all normal chromosomes in HT1080 cells but not on any of the NC-MiCs, including the linear NC-MiC3 (e.g. Figure 4D). This could be due to inability of the technique to detect low levels of telomere sequences and/or the circular nature of the NC-MiCs.

EXAMPLE 13

NC-MiC stability and neocentromere activity

The mitotic stability of NC-MiCs 3, 4 and 5 was assayed following ≥20 cell divisions in culture media with and without selection. BAC E8 was used in FISH experiments to identify the NC-MiCs and 100 cells were scored for each cell line. For both NC-MiC3 and NC-MiC4, similar retention rates of approximately 80% were observed in the presence or

absence of drug selection, suggesting that both chromosome derivatives were stable even in the absence of selection pressure. A retention rate of 36% was initially observed for the NC-MiC5 cell line with selection. Following removal of selection and culturing for 20 divisions, 37% of cells retained NC-MiC5, again suggesting mitotic stability in the absence of selective pressure; the reduced retention rate of NC-MiC5 compared to NC-MiCs 3 and 4 was most likely related to inherent genomic instability seen in this particular cell line.

As NC-MiC5 appeared to be the smallest derivative constructed, and because of the observed background genome instability in the original cell line, the inventors subcloned this line and examined the stability of NC-MiC5 in resulting clones. Two of the subclones (NC-MiC5a and NC-MiC5b) exhibited greatly increased stability of the NC-MiC5. Following ≥50 cell divisions in the absence of drug selection, these two cell lines demonstrated retention rates of 90% and 93%, respectively. These retention rates were not significantly different from those (90% and 91%, respectively) seen in cells passaged for the same number of divisions in the presence of selection. NC-MiC5a cells contained either one copy (76% of cells) or two copies (14% of cells) of the mini-chromosome, while only one copy was consistently detected in the NC-MiC5b cells. The structures of the NC-MiC5a and 5b were shown by detailed FISH analysis to be identical to the original NC-MiC5 chromosome (Figures 1C and 5).

Immunofluorescence detection was used to investigate the functional status of the neocentromeres on the NC-MiCs. Antisera to a host of centromere-associated proteins, including CENP-A, CENP-B, CENP-C, CENP-E, CENP-F, hZW10, p55CDC, and BUB1 were tested. All proteins with the exception of CENP-B were clearly detected on each of the NC-MiCs (some examples are shown in Figures 3F-G, 4F-H, 5E-G). These protein-distribution profiles were indistinguishable from those previously established in the parental mardel(10) chromosome (25,27), confirming that the NC-MiC derivatives contained full neocentromere activity.

EXAMPLE 14

Cell culture and transfection of NC-MiC2

NC-MiC2 was cultured in DMEM (Gibco BRL) with 10% v/v FCS. Hygromycin (Roche) was added to medium at a concentration of 250 μg/ml. Transfection of NC-MiC2 was carried out using electroporation or lipofection. Electroporation was performed (0.4 kV, 250 uF) using a Biorad Gene Pulser Electroporator. For lipofection, the cells were plated one day prior to transfection to give 60-70% confluency at the time of transfection. Two ml of diluted Fugene 6 transfection reagent (100 μl in a total of 2 ml containing 20 μg DNA) (Boehringer Mannheim) was added onto cells drop-wise. The DNA used in transfection was a TACT construct containing human telomeric sequence htel, two loxP sites flanking a neomycin resistance gene, targeting genomic DNA and a blasticidin resistance gene (Figure 7A). The antibiotic selection was applied 24-48 hours post-transfection at the concentration of 250 μg/ml for a period of 14 days before the colonies were picked for further characterization.

EXAMPLE 15

Truncation of p' arm of NC-MiC2

TACT experiments were performed on the p' arm in NC-MiC2 cells using a neomycin-resistance truncation-construct containing a 4 kb targeting DNA derived from the Y13C15 cosmid/B137il BAC, two loxP sites, neomycin resistance gene and a blasticidin resistance gene outside the targeting DNA. A successful targeting event would result in the loss of blasticidin resistance gene. Of 10,000 neomycin-resistant cell lines, approximately 10% were blasticidin sensitive. Sensitivity to blasticidin was determined by culturing the clones in 5 μg/ml of blasticidin. Clones that were neomycin resistant but blasticidin sensitive were subjected to FISH analysis.

EXAMPLE 16

Characterization of NC-MiC6

One cell line (NC-MiC6) appeared to have undergone targeted truncation and was characterized extensively by FISH. Cosmids and BACs proximal to the targeting site were found to be present, whereas all clones distal to this site were absent (summarized in Figure 7C). Based on the inventors' FISH and recently available genome sequence data, the inventors estimated the size of NC-MiC6 to be 1.2 Mb.

No α-satellite (pTRA7) and CENP-B binding were detected on the truncated chromosome. Immunofluorescence using CREST-6 autoimmune serum confirmed neocentromere activity on NC-MiC6. These data support, therefore, a targeted-truncation event that removed most of the p' arm of mardel(10) in NC-MiC2. The mitotic stability of NC-MiC6 was assayed with and without selection for up to 60 divisions in culture. Retention rates of >80% were observed after 20 cell divisions, 75% at 40 cell divisions and with 70% ag 60 divisions (Figure 8) in the absence of selection. In the presence of neomycin, 85% was maintained after 40 divisions and as high as 75% of NC-MiC6 was retained over 60 cell divisions, with loss rate of 0.42% per division, suggesting that NC-MiC6 was mitotically stable over time.

The presence of two loxP sites may be used for excision of neomycin resistance gene and insertion of new gene into specific site using cre-recombinase. The excision and insertion of genes is carried out either *via* transient transfection of plasmid containing the cre-recombinase gene or protein transfection of cre-recombinase.

EXAMPLE 17

Microcell mediated chromosome transfer to mouse embryonic stem ES cells expressing GFP (green fluorescent protein) and to mouse F9 teratocarcinoma cells

Mouse embryonic stem cells were cultured in ES medium (Gibco BRL) with 20% v/v FCS. Mouse F9 cells were cultured in DMEM (Trace Biosciences) with 10% v/v FCS.

Transfection of ES cells was carried out using electroporation at 0.8 kV and 3 uF using a Biorad Gene Pulser Electroporator. The DNA used in transfection was pEGRP-N1 (Clontech) containing the gene encoding the green fluorescent protein and a neomycin resistance gene. Antibiotic selection was applied 24-48 hours post transfection (250 μg/ml G418 neomycin) for a period of 14 days before the colonies were picked and scaled up for screening.

Microcell fusion was carried out as previously described. Log-phase donor ZB30 cells arrested in colcemid for 48 hours were resuspended in percoll/serum-free DMEM (1:1) supplemented with 20 μg/ml of cytochalasin B (Sigma). The cell suspension was then subjected to centrifugation at 18,000 rpm for 90 min at 32°C. Both bands of cell-mix were pelleted, washed with serum-free DMEM and filtered through isopore membranes of 30, 8 and 5 μM (Millipore Corp., MA). Microcells were then fused with recipient neomycin-resistant ES-GFP or F9 cells by addition of 50% w/v PEG (Roche) for 2 min at room temperature. After incubation, cells were rinsed and cultured overnight in ES media containing 20% v/v FCS or DMEM containing 10% v/v FCS followed by addition of antibiotic selection (250 μg/ml G418 neomycin and 100 μg/ml of zeocin for ES cells and 100 μg/ml zeocin for F9 cells) 24 hrs later.

EXAMPLE 18

Characterization of ES-GFPmar (10)#1 and F9-4-5mar(10) cell lines

Extensive FISH analysis of ES-GFPmar(10)#1 and F9-4-5mar(1) demonstrated that the mardel(10) chromosome and the neocentromere contained therein were intact. No mouse centromeric/pericentromeric major and minor satellite DNA or genomic DNA was detected on the marker chromosome in either cell line. In addition, mardel(10) was the only human chromosome present in these cell lines. The stability of mardel(10) was assayed with selection for up to 45 divisions in culture and the marker chromosome showed mitotic stability in both cell lines.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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CLAIMS

1. An isolated nucleic acid molecule comprising a sequence of nucleotides derived from a eukaryotic chromosome and encompassing a neocentromere which nucleic acid molecule exists in a truncated or modified form in a compatible cell or, when introduced by transfection as a pre-fabricated DNA entity into a compatible cell, is capable of replicating, acting as an extra chromosomal element and segregating with cell division.

2. An isolated nucleic acid molecule of Claim 1 wherein the eukaryotic chromosome is derived from a mammal including a human or primate or plant, avian species, insect, worm, fungus, yeast or reptile.

3. An isolated nucleic acid molecule of Claim 2 wherein the eukaryotic chromosome is derived from a human.

4. An isolated nucleic acid molecule of Claim 2 wherein the eukaryotic chromosome is derived from a livestock animal.

5. An isolated nucleic acid molecule of Claim 1 wherein the molecule is generated by a combination of targeted telomere-associated truncation of a q' arm and a random truncation of a p' arm of the mardel(10) chromosome or its equivalent.

6. An isolated nucleic acid molecule of Claim 5 wherein said nucleic acid molecule comprises a neocentromere at a location equivalent to between q24 and q26 on chromosome 10 or its equivalent.

7. An isolated nucleic acid molecule of Claim 6 wherein said nucleic acid molecule comprises a neocentromere at a location equivalent to at or about q25 on chromosome 10 or its equivalent.

8. An isolated nucleic acid molecule of Claim 7 wherein said nucleic acid molecule comprises a neocentromere at a location equivalent to at or about q25.2 on chromosome 10 or its equivalent.

9. A nucleic acid molecule of Claim 1 or 6 or 7 or 8 wherein the region corresponding to a neocentromere is substantially devoid of α-satellite DNA.

10. A nucleic acid molecule of Claim 9 wherein the nucleic acid molecule is from about 0.5 to about 2.0 Mb in size.

11. A nucleic acid molecule of Claim 10 wherein the nucleic acid molecule is from about 0.8 to about 1.6 Mb in size.

12. A method for identifying a neocentromere or a functional homolog, said method comprising isolating DNA by chromatin immunoprecipitation using an antibody specific to mammalian CENP-A and/or CENP-C or an antibody capable of cross interacting with mammalian CENP-A and/or CENP-C, amplifying the DNA isolated by immunoprecipitation and incorporating label into the amplified DNA and then using the amplified DNA to probe a DNA array comprising genomic DNA or its equivalent and identifying and isolating clones which hybridize to said immunoprecipitated DNA.

13. A method of Claim 12 wherein the mammal is a human, livestock animal, companion animal or laboratory test animal.

14. A method of Claim 13 wherein the mammal is a human.

15. A method of Claim 14 wherein the neocentromere is at a location equivalent to between p24 and p26 on chromosome 10 or its equivalent.

16. A method of Claim 15 wherein the neocentromere is at a location at or about q25 on chromosome 10 or its equivalent.

17. A method of Claim 16 wherein the neocentromere is at a location at or about q25.2 on chromosome 10 or its equivalent.

18. An isolated nucleic acid molecule in the form of a human neocentromere-based mini-chromosome (NC-MiCs), said NC-MiCs comprising a neocentromere or a latent, synthetic or hybrid form thereof which enable stable segregation during cell division.

19. An isolated nucleic acid molecule of Claim 18 generated by a combination of targeted telomere-associated truncation of a q' arm and a random truncation of a p' arm of the mardel(10) chromosome or its equivalent.

20. An isolated nucleic acid molecule of Claim 19 wherein the neocentromere is at a location equivalent to between q24 and q26 on chromosome 10 or its equivalent.

21. An isolated nucleic acid molecule of Claim 20 wherein the neocentromere is at a location at or about q25 on chromosome 10 or its equivalent.

22. An isolated nucleic acid molecule of Claim 20 wherein the neocentromere is at a location at or about q25.2 on chromosome 10 or its equivalent.

23. A method for generating a mini-chromosome, said method comprising:-

introducing into a human or mammalian cell which carries a chromosome containing a neocentromere, a truncation construct comprising a vector having telomeric sequences, a selectable marker, homologous targeting DNA on one or other of the q' or p' arm of the target chromosome;

selecting for cells expressing the selectable marker;

introducing into said cells a second truncation construct comprising the other of said q' or p' targeting DNA;

selecting for cells expressing the selectable marker associated with said second truncation construct; and

then isolating the truncated chromosome which comprises a neocentromere.

24. A method of Claim 23 wherein the cell is a human cell.

25. A method of Claim 24 wherein the neocentromere is at a location equivalent to between q24 and q26 on chromosome 10 or its equivalent.

26. A method of Claim 24 wherein the neocentromere is at a location at or about q25 on chromosome 10 or its equivalent.

27. A method of Claim 24 wherein the neocentromere is at a location at or about q25.2 on chromosome 10 or its equivalent.

28. An isolated cell comprising a chromosome having q' and p' arms flanking a neocentromere wherein one or both q' and/or p' arms are truncated.

29. An isolated cell line deposited at ECAAC under Accession 00122001 (CHO/BE ZB30).

30. An isolated cell line deposited at ECAAC under Accession 00122002 (HT1080- MIC 1).

31. An isolated cell line deposited at ECAAC under Accession 00122003 (HT1080- MIC 2).

32. An isolated cell line deposited at ECAAC under Accession 00122004 (HT1080- MIC 3).

33. An isolated cell line deposited at ECAAC under Accession 00122005 (HT1080- MIC 4).

34. An isolated cell line deposited at ECAAC under Accession 00122006 (HT1080- MIC 5).

35. An isolated cell line deposited at ECAAC under Accession 00122007 (HT1080- MIC 5a).

36. An isolated cell line deposited at ECAAC under Accession 00122008 (HT1080- MIC 5b).

37. Use of an isolated nucleic acid molecule according to Claim 1 in the manufacture of a mini-chromosome for use in gene therapy.

38. Use of Claim 37 wherein the mini-chromosome is an NC-MiC.

39. Use of Claim 37 wherein the mini-chromosome is a YAC.

40. Use of Claim 37 wherein the mini-chromosome is a HAC.

41. Use of Claim 37 wherein the mini-chromosome is a MAC.

42. Use of Claim 37 wherein the mini-chromosome is a PLAC.

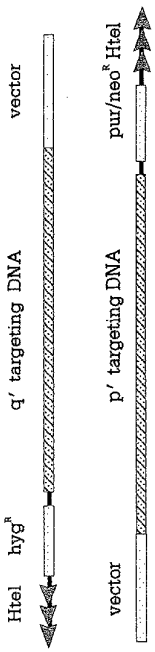

HtelI
hyg^R
q′ targeting DNA
vector
vector
p′ targeting DNA
pur/neo^R HtelI

Figure 1A

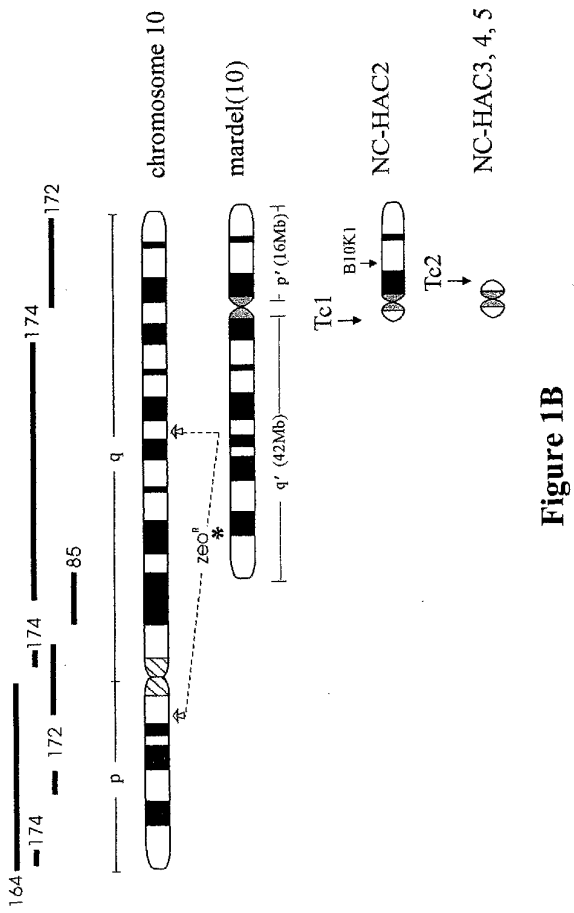

chromosome 10
mardel(10)
NC-HAC2
NC-HAC3, 4, 5
164
174
172
174
85
172
174
p
q
zeo^R
q' (42Mb)
p' (16Mb)
Tc1
B10K1
Tc2

Figure 1B

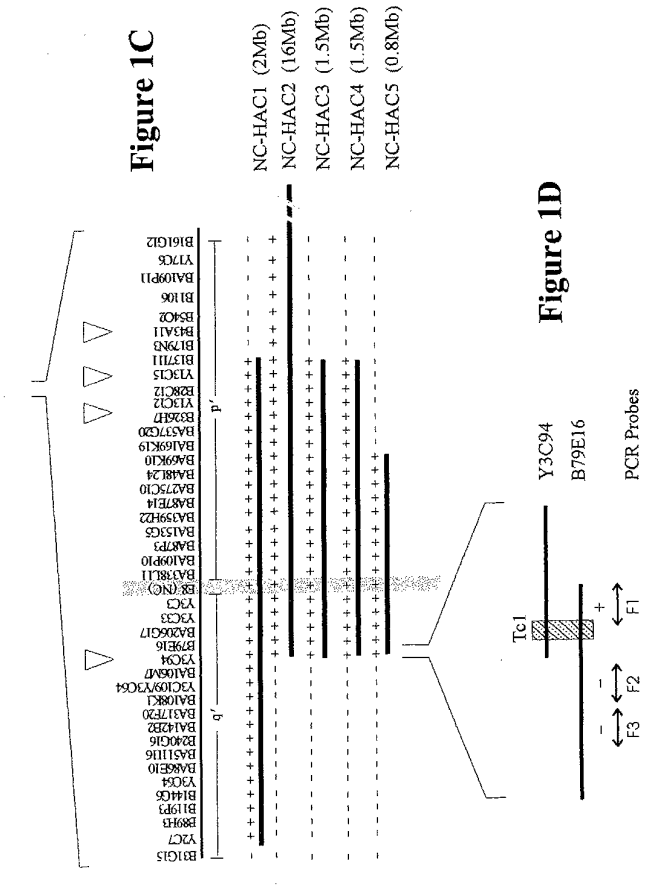


Figure 1C
NC-HAC1 (2Mb)
NC-HAC2 (16Mb)
NC-HAC3 (1.5Mb)
NC-HAC4 (1.5Mb)
NC-HAC5 (0.8Mb)
p'
q'
Figure 1D
Y3C94
B79E16
PCR Probes
Tc1
F1
F2
F3

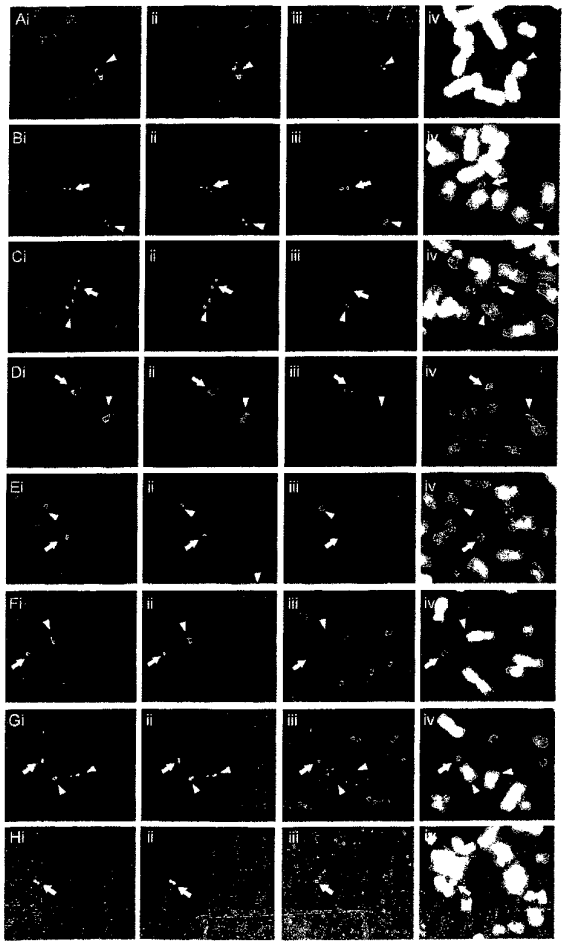
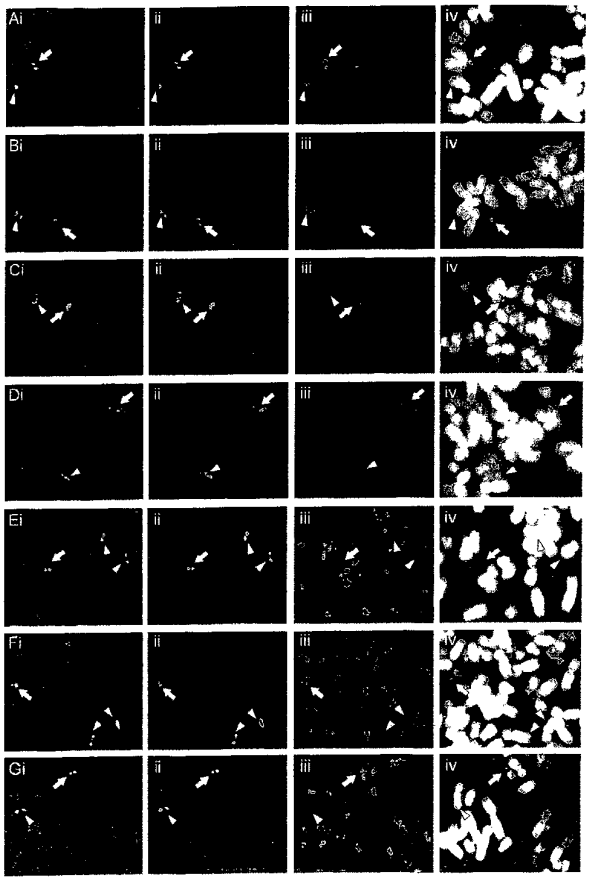

Ai
ii
iii
iv
Bi
ii
iii
iv
Ci
ii
iii
iv
Di
ii
iii
iv
Ei
ii
iii
iv
Fi
ii
iii
iv
Gi
ii
iii
iv
Hi
ii
iii
iv

Figure 2


Ai
ii
iii
iv
Bi
ii
iii
iv
Ci
ii
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Fi
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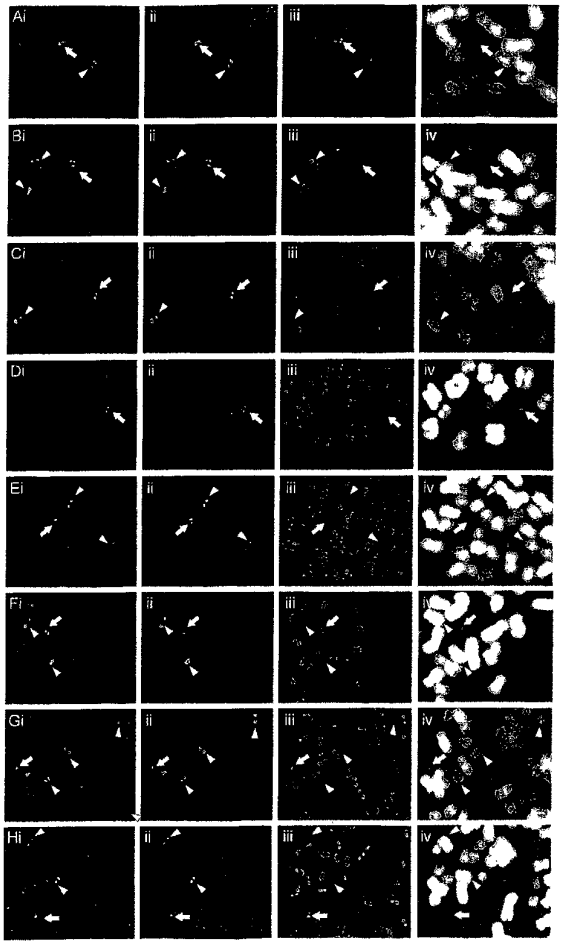
iii
iv

Figure 3


Ai
ii
iii
Bi
ii
iii
iv
Ci
ii
iii
iv
Di
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iii
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Ei
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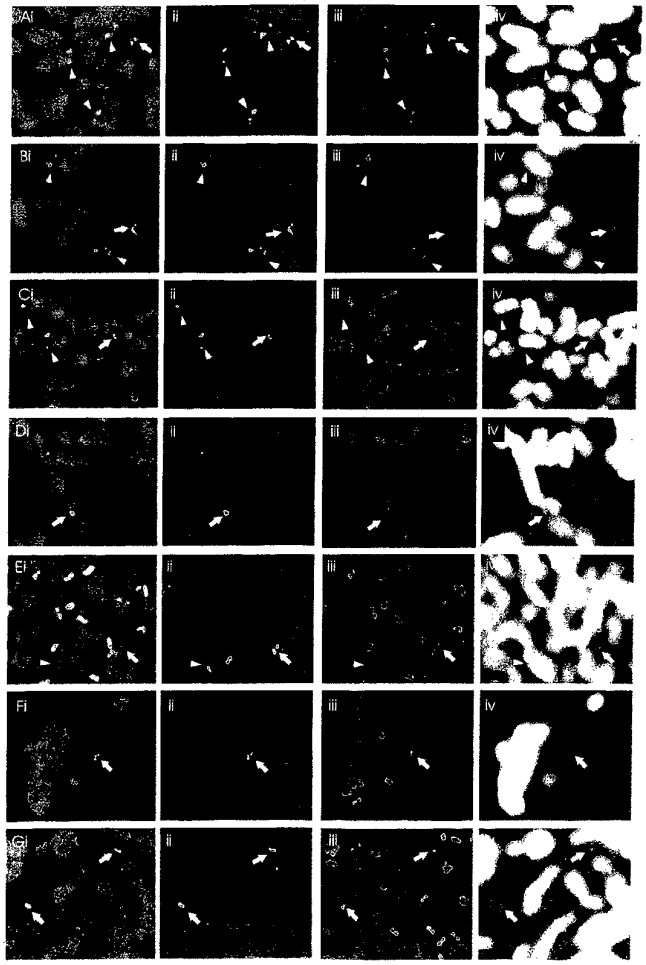
Fi
ii
iii
Gi
ii
iii
iv
Hi
ii
iii
iv

Figure 4


Ai
ii
iii
iv
Bi
ii
iii
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Ci
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Di
ii
iii
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Ei
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iii
Fi
ii
iii
iv
Gi
ii
iii

Figure 5

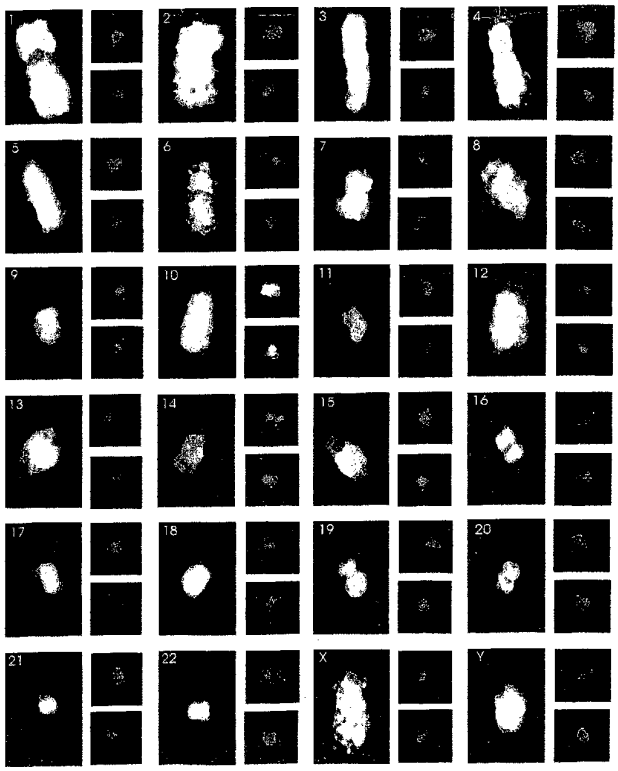

Figure 6A

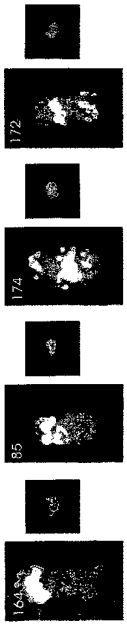

Figure 6B

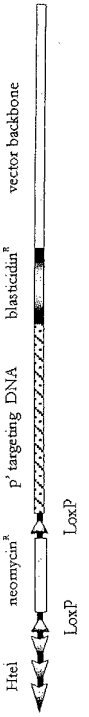

Figure 7A

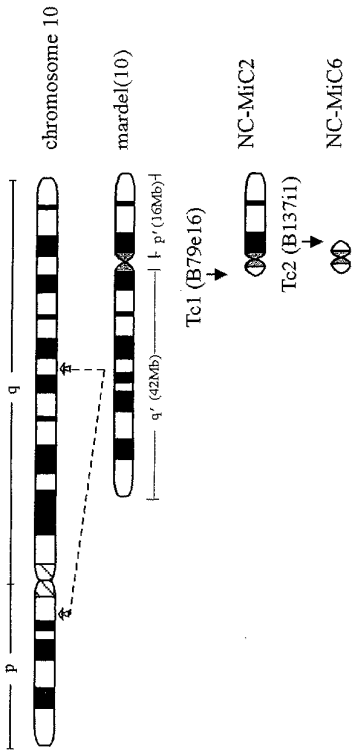
chromosome 10
mardel(10)
NC-MiC2
NC-MiC6
p
q
q' (42Mb)
p' (16Mb)
Tc1 (B79e16)
Tc2 (B137i1)

Figure 7B

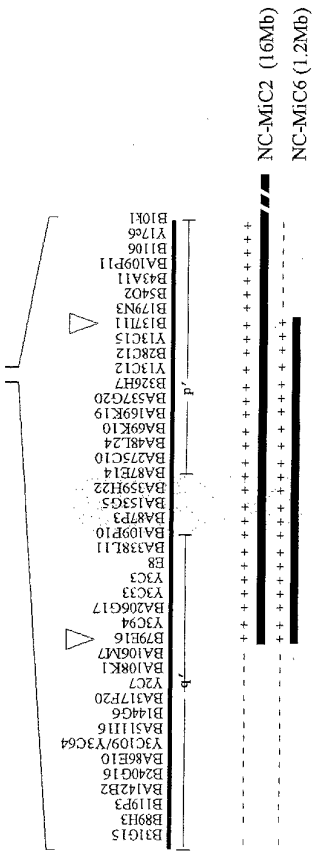
NC-MiC2 (16Mb)
NC-MiC6 (1.2Mb)
B310I5
B89H3
BA142B2
B119P3
B240G16
BA866E10
Y3C109/Y3C64
BA51111G
B144G6
BA317P20
Y2C7
BA108K1
BA106M7
B79E16
Y3C94
BA206G17
Y3C33
Y3C3
E8
BA133L11
BA109P10
BA87P3
BA153G5
BA359H22
BA87E14
BA275C10
BA48L24
BA69K10
BA169K19
BA537G20
B326H7
Y13C12
B28C12
Y13C15
B137I11
B179N3
B540Z
B43A11
BA109P11
B1106
Y1766
B10K1
p'
q'

Figure 7C

cell line	passage no	division no	Drug selection	No of cells scored	percentages of retention	loss per division
MIC-6	5	10	Neomycin	20	19/20 (95%)	0.50%
			None	20	18/20 (90%)	1.00%
	10	20	Neomycin	20	17/20 (85%)	0.75%
			None	20	16/20 (80%)	1.0%
	20	40	Neomycin	20	16/20 (80%)	0.5%
			None	20	15/20 (75%)	0.625%
	30	60	Neomycin	20	15/20(75%)	0.42%
			None	20	14/20(70%)	0.5%

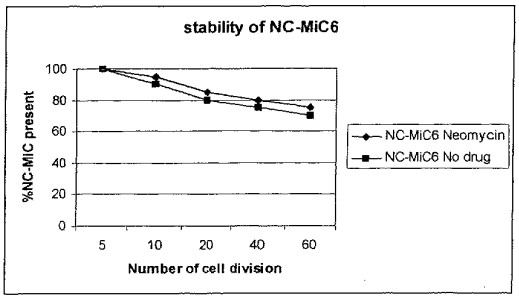

Figure 8

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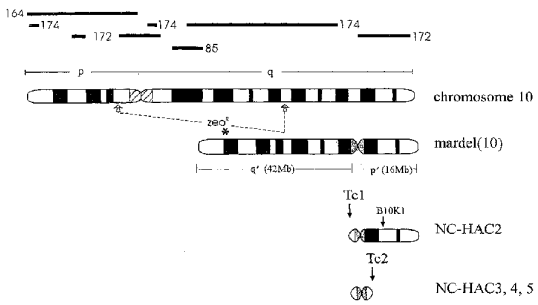
[Continued on next page]

(54) Title: NEOCENTROMERE-BASED MINI-CHROMOSOMES OR ARTIFICIAL CHROMOSOMES



(57) Abstract: The present invention is directed generally to a defined or isolated nucleic acid molecule encompassing a neocentromere or a functional derivative thereof or a latent, synthetic or hybrid form thereof and its use *inter alia* in developing a range of eukaryotic mini-chromosomes and artificial chromosomes including mammalian (e.g. human) and non-mammalian mini-chromosomes and artificial chromosomes. The present invention further provides a telomere-associated chromosome truncation (TACT) approach to develop mini-chromosomes, but is not limited to this approach. It is directed to any truncation approach that produces a neocentromere-containing mini-chromosome, or any transfection approach using cloned or pre-fabricated DNA that provides neocentromere function in the construction of artificial chromosome. Such mini-chromosomes and artificial chromosomes are useful in a range of genetic therapies.

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NEOCENTROMERE-BASED MINI-CHROMOSOMES OR ARTIFICIAL CHROMOSOMES

FIELD OF THE INVENTION

The present invention is directed generally to a defined or isolated nucleic acid molecule encompassing a neocentreomere or a functional derivative thereof or a latent, synthetic or hybrid form thereof and its use *inter alia* in developing a range of eukaryotic mini-chromosomes and artificial chromosomes including mammalian (e.g. human) and non-mammalian mini-chromosomes and artificial chromosomes. The present invention further provides a telomere-associated chromosome truncation (TACT) approach to develop mini-chromosomes, but is not limited to this approach. It is directed to any truncation approach that produces a neocentromere-containing mini-chromosome, or any transfection approach using cloned or pre-fabricated DNA that provides neocentromere function in the construction of artificial chromosome. Such mini-chromosomes and artificial chromosomes are useful in a range of genetic therapies.

BACKGROUND OF THE INVENTION

Bibliographic details of the publications numerically referred to in this specification are collected at the end of the description.

Reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in Australia or any other country.

The rapidly increasing sophistication of recombinant DNA technology is greatly facilitating research and development in the medical and allied health fields. A particularly important area is in mammalian including human genetics and the elucidation of the molecular mechanisms behind genetic abnormalities. Progress in research in this area has been hampered by the lack of a fully sequenced nucleic acid molecule containing a human

centromere. The identification and/or cloning of such a molecule would promote the development of techniques for introducing genes into eukaryotic cells and in particular mammalian including human cells and will be an important asset to gene therapy and the development of expression systems for the production of a range of recombinant gene products in mammalian cells. Importantly, the identification and/or cloning of a fully sequenced centromeres facilitates the development of mammalian mini-chromosomes and artificial chromosomes.

Mammalian mini-chromosomes and artificial chromosomes have a variety of potential biotechnological and therapeutic applications arising from their ability to exist episomally and allow expression of genes under their endogenous control elements independently of the host genomic DNA. Because they are in effect fully functional mammalian chromosomes, there is no theoretically upper limit to the size of DNA that can be introduced into these entities. By analogy with their yeast counterparts, it has been assumed that mammalian mini-chromosomes and artificial chromosomes require a functional mammalian centromere, telomeres and DNA replication origins in order for proper segregation. At present, the least understood and most complex of these three components is the centromere.

The identification of an increasing number of protein components necessary for correct centromere activity, and the characterization of centromere DNA sequences in a variety of species, have greatly increased the knowledge of the mechanisms underlying centromere formation and function (1,2,3). This knowledge has facilitated the development of a number of strategies for mammalian mini-chromosome and artificial chromosome construction. One strategy involves the *de novo* formation of human artificial chromosomes by co-transfection of telomeric DNA with large arrays of human α-satellite into human cells (4,5,6,7). Studies using this strategy have shown that only α-satellite DNA-containing CENP-B boxes can participate in *de novo* artificial chromosome formation (5,8). While some of the generated artificial chromosomes were linear in structure (4), others were consistently circular (5,7,8). The artificial chromosomes ranged in size from ~1-13 Mb and were typically one or more orders of magnitude larger than the

input DNA. This increase in size has been attributed to end-joining of input DNA following transfection (4).

A different strategy involves the use of telomere-associated chromosome truncation to remove non-essential chromosomal materials around a normal centromere to produce a mini-chromosome *in situ*. Sequential truncation of a human X chromosome has yielded a 2.5-Mb mini-chromosome comprising approximately 1.8 Mb of X-chromosome α-satellite DNA and 400 kb of proximal Xp DNA (9,10,11). This chromosome shows mitotic stability comparable to that of the normal human X chromosome (10,11). A similar approach has produced a number of human Y chromosome-derived mini-chromosomes, ranging in size from ~0.7 Mb to over 4Mb, with the smaller ones being relatively unstable (12,13). The larger mini-chromosomes were stably maintained in CHO cells, chicken DT40 cells and mouse L cells but showed poor stability when introduced into mouse ES cells, suggesting differential requirements for correct centromere function in different cell types (14,15).

A third strategy for production of mammalian artificial chromosomes involves the amplification of pericentric DNA followed by controlled breakage of chromosomes to produce satellite DNA-based artificial chromosomes of between 60 and 400 Mb (16,17,18,19,20).

In recent years, neocentromeres (NCs) that lack the repeat sequences traditionally associated with centromere function have been described in humans (21) and *Drosophila* (22). Characterization of the underlying DNA and protein-binding profile of neocentromeres in humans has suggested epigenetic mechanism of centromere formation independent of primary DNA sequence composition (23,24,25,26). The discovery of neocentromeres provides an alternative approach to the construction of mini-chromosomes and artificial chromosomes to those previously described that have been based on the use of repeated centromere DNA sequences.

In accordance with the present invention, the inventors have generated a series of

mitotically stable, human mini-chromosomes containing a fully functional human neocentromere. The generation of a human mini-chromosome permits the development of genetic therapies, transgenic plant and animal production and recombinant protein production.

SUMMARY OF THE INVENTION

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

Nucleotide and amino acid sequences are referred to by a sequence identifier number (SEQ ID NO:). The SEQ ID NOs: correspond numerically to the sequence identifiers <400>1, <400>2, etc. A sequence listing is provided after the claims.

The present invention is predicated in part on the use of a telomere-associated chromosome truncation (TACT) approach to develop mini-chromosomes. Truncation constructs are developed comprising a selectable marker, a targeting DNA sequence homologous to a small region on the p' or q' arms of a eukaryotic chromosome such as human mardel(10), and a small array of telomeric sequences. p' and q' arms refer to the short and long arms of the mardel(10) chromosome but also refer generally to the short and long arms of any marker chromosome containing a neocentromere. A first truncation construct is transfected into a target cell and the marker selected. This results in a target chromosome with a truncated p' or q' arm depending on the truncation construct used. A second truncation construct is then transfected into the same cell employing the other of the p' or q' arm truncation construct. Again, following selection, a truncated form of the p' or q' arm of the target chromosome is obtained. The resulting mini-chromosome may then be isolated and used for gene therapy or gene expression. A modified targeting method that may or may not include a telomere DNA may also be used to introduce genes or other nucleotide sequences into a target chromosome.

One aspect of the present invention provides an isolated nucleic acid molecule comprising a sequence of nucleotides which defines an eukaryotic neocentromere.

More particularly, the present invention provides an isolated nucleic acid molecule

comprising a sequence of nucleotides derived from a eukaryotic chromosome and encompassing a neocentromere which nucleic acid molecule exists in a truncated or modified form in a compatible cell or, when introduced by transfection as a pre-fabricated DNA entity into a compatible cell, is capable of replicating, acting as an extra chromosomal element and segregating with cell division.

Another aspect of the present invention contemplates the use of a method for identifying a neocentromere or a functional homolog, said method comprising isolating DNA by chromatin immunoprecipitation using an antibody specific to mammalian CENP-A and/or CENP-C or an antibody capable of cross interacting with mammalian CENP-A and/or CENP-C, amplifying the DNA isolated by immunoprecipitation and incorporating label into the amplified DNA and then using the amplified DNA to probe a DNA array comprising genomic DNA or its equivalent and identifying and isolating clones which hybridize to said immunoprecipitated DNA.

A further aspect of the present invention provides an isolated nucleic acid molecule in the form of a human neocentromere-based mini-chromosome (NC-MiCs) comprising a neocentromere or a latent, synthetic or hybrid form thereof which enable stable segregation during cell division.

Yet another aspect of the present invention provides a method for generating a mini-chromosome, said method comprising:-

introducing into a human or mammalian cell which carries a chromosome containing a neocentromere, a truncation construct comprising a vector having telomeric sequences, a selectable marker, homologous targeting DNA on one or other of the q' or p' arm of the target chromosome;

selecting for cells expressing the selectable marker;

introducing into said cells a second truncation construct comprising the

other of said q' or p' targeting DNA;

selecting for cells expressing the selectable marker associated with said second truncation construct; and

then isolating the truncated chromosome which comprises a neocentromere.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a diagrammatic representation showing neocentromere-based mini-chromosomes (abbreviated as NC-MiC) formation from the mardel(10) chromosome. **(A)** Structure of TACT targeting constructs. Targeting DNA from the p' or q' arms of mardel(10) were cloned into vectors containing small arrays of cloned human telomeric DNA (HTel) adjacent to a mammalian selectable marker (hygromycin^R, neomycin^R, or puromycin^R). Constructs were linearized at a restriction site between the vector DNA and the telomere repeats to expose the telomere sequences at the terminal. Following a homologous recombination event, the vector DNA should not be incorporated into the host chromosome. Loss of vector DNA allowed design of a screening assay for possible recombination events. **(B)** Schematic formation of mardel(10) and NC-MiCs. Open arrows indicate the breakpoints on the normal chromosome 10 in the generation of mardel(10). The long and short arms of mardel(10) are denoted as q' and p', respectively. The truncation events resulting in the formation of NC-MiCs 1-5 are represented by Tc1 and Tc2. Location of the zeocin resistance gene inserted into mardel(10) is indicated by an asterisk. Position of BAC B10K1 used in a screening assay for loss of p' arm is shown. Bold lines above chromosome 10 denote the locations and designations of sub-chromosomal DNA paints used to characterize the NC-MiCs. **(C)** Mapping of the NC-MiCs. Ordered cosmid and BAC clones covering approximately 3 Mb and previously mapped to the 10q25 neocentromere region are shown. Vertical shaded area represents the E8 BAC containing the centromere protein-binding neocentromere (NC) domain (23,27). Open arrowheads to the left and right of E8 indicate intended positions of targeted truncation using q' and p' truncation constructs. (+) denotes a positive FISH result for a BAC or cosmid probe on an NC-MiC, while (–) indicates a negative FISH result. The approximate sizes of the different NC-MiCs are shown in parentheses. **(D)** Characterization of the targeted q' truncation site. The targeting DNA for q' truncation is a 6-kb *Xba*I fragment subcloned from cosmid Y3C94 (also present in BAC B79E16) and is represented by the hatched box. Locations of PCR probes from B79E16 are indicated by double-headed arrows and are denoted F1, F2, and F3. Hybridization status of these probes

on NC-MiCs is denoted by (+) or (–) confirming that the q' truncation was the result of a targeted event.

Figure 2 is a photographic representation of FISH and/or immunofluorescence analysis of ZB30 and NC-MiC2. **(A)** FISH analysis of ZB30 showing hybridization of E8 (green) and tagging of mardel(10) (arrowhead) with zeocin resistance gene (red). **(B-H)** FISH and/or immunofluorescence analysis on NC-MiC2. Normal chromosome 10 is indicated by arrowhead and NC-MiC2 by arrow. (i-iv) Combined image, and split images for green, red, and DAPI, respectively. **(B)** FISH using E8 (green) and Y3C94 cosmid probe (red), showing presence of Y3C94 on NC-MiC2. **(C)** FISH using E8 (green) and q' cosmid Y3C109 (red), showing absence of Y3C109 on NC-MiC2. **(D)** FISH using E8 (green) and a PCR fragment F1 (red) derived from Y3C94/B79E16 and overlapping the q' targeting DNA (see Figure 1D), showing presence of F1 DNA on NC-MiC2. **(E)** FISH using E8 (green) and a PCR fragment F2 (red) derived from B79E16 in a region immediately distal to the targeting DNA (see Figure 1D), showing absence of F2 DNA on NC-MiC2. **(F)** FISH using E8 (green) and immunofluorescence using anti-CENP-B antibody (red), showing absence of CENP-B on NC-MiC2. **(G)** FISH using E8 (green) and immunofluorescence using anti-CENP-E antibody (red), showing presence of CENP-E protein on NC-MiC2. **(H)** FISH using E8 (green) and telomere-repeat PNA probe (red), showing telomeric sequences on NC-MiC2.

Figure 3 is a photographic representation of FISH and/or immunofluorescence analysis of NC-MiC3. Normal chromosome 10 is indicated by arrowhead and NC-MiC3 by arrow. (i-iv) Combined image, and split images for green, red, and DAPI, respectively. **(A)** FISH using E8 (green) and q' BAC B79E16 (red), showing presence of B79E16 on NC-MiC3. **(B)** FISH using E8 (green) and q' cosmid Y3C109 (red), showing absence of Y3C109 on NC-MiC3. **(C)** FISH using E8 (green) and p' cosmid Y13C12 (red), showing presence of Y13C12 on NC-MiC3. **(D)** FISH using E8 (green) and p' BAC B179N3 (red), showing absence of B179N3 on NC-MiC3. **(E)** FISH using E8 (green) and a pan-α-satellite pTRA-7 probe (red), showing absence of α-satellite on NC-MiC3. **(F)** FISH using E8 (green) and immunofluorescence using anti-CENP-E antibody (red), showing presence of CENP-E on

NC-MiC3. **(G)** FISH using E8 (green) and immunofluorescence using anti-CENP-F antibody (red), showing presence of CENP-F on NC-MiC3.

Figure 4 is a photographic representation of FISH and/or immunofluorescence analysis of NC-MiC4. Normal chromosome 10 is indicated by arrowhead and NC-MiC4 by arrow. (i-iv) Combined image, and split images for green, red, and DAPI, respectively. **(A)** FISH using E8 (green) and p' cosmid Y13C12 (red), showing the presence of Y13C12 on NC-MiC4. **(B)** FISH using E8 (green) and p' BAC B43A11 (red), showing the absence of B43A11 on NC-MiC4. **(C)** FISH using E8 (green) and pan-α-satellite pTRA-7 probe (red), showing absence of α-satellite on NC-MiC4. **(D)** FISH using TTAGGG telomere-repeat PNA probe, showing positive signals on all human telomeres but not on NC-MiC4. **(E)** FISH using E8 (green) and immunofluorescence using anti-CENP-B antibody (red), showing absence of CENP-B on NC-MiC4. **(F)** FISH using E8 (green) and immunofluorescence using anti-CENP-C antibody (red), showing presence of CENP-C on NC-MiC4. **(G)** FISH using E8 (green) and immunofluorescence using anti-CENP-E antibody (red), showing presence of CENP-E on NC-MiC4. **(H)** FISH using E8 (green) and immunofluorescence using anti-CENP-F antibody (red), showing presence of CENP-F on NC-MiC4.

Figure 5 is a photographic representation of FISH and/or immunofluorescence analysis of NC-MiC5. Normal chromosome 10 is indicated by arrowhead and NC-MiC5 by arrow. (i-iv) Combined image, and split images for green, red, and DAPI, respectively. **(A)** FISH using E8 (green) and p' BAC BA48L24 (red), showing presence of BA48L24 on NC-MiC5. **(B)** FISH using E8 (green) and p' BAC BA69K10 (red), showing absence of BA69K10 on NC-MiC5. **(C)** FISH using E8 (green) and pan-α-satellite pTRA-7 probe (red), showing absence of α-satellite on NC-MiC5. **(D)** FISH using E8 (green) and immunofluorescence using anti-CENP-B antibody (red), showing absence of CENP-B on NC-MiC5. **(E)** FISH using E8 (green) and immunofluorescence using anti-CENP-A antibody (red), showing presence of CENP-A on NC-MiC5. **(F)** FISH using E8 (green) and immunofluorescence using anti-CENP-C antibody (red), showing presence of CENP-

C on NC-MiC5. **(G)** FISH using E8 (green) and immunofluorescence using anti-CENP-E antibody (red), showing presence of CENP-E on NC-MiC5.

Figure 6 is a photographic representation of chromosome-painting analysis of NC-MiCs. **(A)** Whole-chromosome paints for all 24 human chromosomes (1-22, X, Y). Left panel shows positive painting (green) on normal human chromosomes, except for the pericentric heterochromatic regions and those on the short arms of acrocentric chromosomes, and the q12 region of Y. Upper right panel shows the corresponding painting result for NC-MiC4 while lower right panel shows the result for NC-MiC5. All chromosome paints with the exception of chromosome 10 are negative on the NC-MiCs as evidenced by a lack of overlapping paint (green) and E8 (red) signals (confirmed by splitting individual images; not shown), while the chromosome-10 paint signal is clearly seen on NC-MiCs as evidenced by an overlap of red and green signals producing a combined yellow colour. **(B)** Subchromosome-10 paints on NC-MiC5. Positions for these subchromosome paints on chromosome 10 are shown schematically in Figure 1B. Left-hand panels show designations of the paints and FISH results (green) on normal chromosome 10. Right-hand panels show the negative results obtained for these paints on NC-MiC5. E8 signals are in red. Chromosomes and NC-MiCs are not shown to scale.

Figures 7A-C are diagrammatic representations showing NC-MiC6 construction *via* the truncation of NC-MiC2. **(A)** Structure of TACT targeting constructs used for truncating the p' arm from NC-MiC2. Targeting DNA from the p' arm of mardel(10) and a mammalian selectable marker (blasticidin resistance gene, blasticidin^R) were cloned into vectors containing small arrays of cloned human telomeric DNA (Htel) adjacent to an antibiotic resistance gene, neomycin^R. Constructs were linearized at a restriction site between the vector DNA and the telomere repeats to expose the telomere sequences at the terminal. Following a homologous recombination event, the blasticidin resistance gene should not be incorporated into the host chromosome. Loss of the blasticidin resistance gene allowed the design of a screening assay for possible recombination events. **(B)** Schematic formation of mardel(10) and NC-MiCs. Open arrows indicate the breakpoints on the normal chromosome 10 in the generation of mardel(10). The long and short arms of mardel(10)

are denoted as q' and p', respectively. NC-MiC2 was truncated at B79e16/Y3C94 (Tc1) (as described earlier) from mardel(10), whereas NC-MiC6 is a result of truncation 2, Tc2, at B137iL/Y13c15 using the TACT construct described in (A). **(C)** Mapping of the NC-MiCs 2 and 6. Ordered cosmid and BAC clones covering approximately 3 Mb and previously mapped to the 10q25 neocentromere region are shown. the vertical shaded area represents the centromere protein CENP-A-binding domain (51). Open arrowheads indicate positions of targeted truncation. (+) denotes a positive FISH result for a BAC or cosmid probe on an NC-MiC, while (-) indicates a negative FISH result. The approximate sizes of the different NC-MiCs are shown in parentheses.

Figure 8 is a tabular and graphical representation showing the results of culturing NC-MiC6 for 60 divisions either in the presence or absence of neomycin drug G418 at a concentration of 150 µg/ml before they were harvested at various intervals for determination of stability. The X axis represents the number of divisions, whereas the Y axis represents the percentage of NC-MiC present in culture as determined by FISH analysis. The results indicated mitotic stability of NC-MiC-6 in the presence or absence of G418.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is predicated in part on the identification and isolation of nucleic acid molecules exhibiting neocentromeric properties. In accordance with the present invention, a neocentromere is considered a centromere which does not contain substantial amounts of repetitive DNA sequences that are present on a normal centromere (e.g. α-satellite in humans and minor satellite in mouse) and, when activated, is capable of functioning as a centromere. For example, a mammalian (e.g. human) neocentromere is a centromere which does not contain substantial α-satellite DNA repeat sequences. The term "substantial" in this context means that the nucleic acid molecule does not contain detectable normal centromeric repetitive DNA sequences such as α-satellite by FISH analysis under medium stringency conditions or by direct sequence comparison under medium homology criteria. The neocentromere may, however, contain a small number of highly diverged normal centromeric repetitive DNA sequences. In primates, for example, α-satellite DNA is considered to be about 170 bp in length. An nucleic acid molecule containing an activated neocentromere or a neocentromere otherwise functioning as a centromere facilitates in accordance with the present invention, the nucleic acid molecule in the form of a mini-chromosome or pre-fabricated with other DNA to facilitate transfection, replicating, remaining extra-chromosomal and segregating with cell division. Reference herein to "neocentromere" is taken to mean a centromere substantially devoid of repetitive DNA sequences that are normally present on the centromere of an endogenous and structurally unaltered chromosome such as α-satellite DNA repeat sequences on human chromosomes. Furthermore, a neocentromere is considered to be derived from a normally non-centromeric region of the genome.

Accordingly, one aspect of the present invention provides an isolated nucleic acid molecule comprising a sequence of nucleotides which defines an eukaryotic neocentromere.

More particularly, the present invention provides an isolated nucleic acid molecule comprising a sequence of nucleotides derived from a eukaryotic chromosome and

encompassing a neocentromere which nucleic acid molecule exists in a truncated or modified form in a compatible cell or, when introduced by transfection as a pre-fabricated DNA entity into a compatible cell, is capable of replicating, acting as an extra chromosomal element and segregating with cell division.

The present invention is exemplified herein by the identification of a human neocentromere. This is done, however, with the understanding that the present invention extends to all eukaryotic neocentromeres such as from mammalian, plant, avian, insect, worm, fungal, yeasts and reptilian chromosomes. The most preferred neocentromere, however, is from human chromosomes and their mammalian homologs.

The present invention is predicated in part on the construction of a number of neocentromere-based human mini-chromosomes using a combination of targeted telomere-associated truncation of the q' arm and apparently random truncation of the p' arm of the mardel(10) chromosome. The latter refers to a chromosome identified in a human patient and results from a re-arrangement of human chromosome 10. The mardel(10) marker is mitotically stable and, in accordance with the present invention, contains a functional neocentromere at a location regarded as non-centromeric. The neocentromere at mardel(10) is located between q24 and q26 on chromosome 10 and more particularly around q25. Even more particularly, the neocentromere maps to q25.2 on chromosome 10. The present invention is exemplified by DNA cloned from the q24-q26 region of the mardel(10) chromosome as well as the corresponding region on normal human chromosome 10. These DNA molecules contain a functional neocentromere. The present invention extends, however, to any neocentromere on any chromosome in mammalian and non-mammalian animals as well as plants, insects, worms, fungal, reptiles and yeasts.

Detailed mapping using cosmid and BAC probes from the 10q25 region allowed the truncation sites to be defined and the demonstration that the NC-MiCs contain single-copy intact DNA from this region. Extensive FISH using pan-α-satellite DNA, whole-chromosome paints for all human chromosomes and different sub-chromosomes-10 paints, revealed that none of the NC-MiCs have acquired detectable amounts of α-satellite DNA

or other human genomic sequences. Based on the mapping data, the sizes of NC-MiCs 3, 4 and 5, were estimated to be approximately 1.6, 1.6 and 0.8 Mb, respectively.

The present invention further contemplates a nucleic acid molecule or its chemical equivalent having a tertiary structure which defines a human neocentromere or a functional derivative thereof or a latent, synthetic or hybrid form thereof or its mammalian or non-mammalian homolog.

Even more particularly, the present invention is directed to an isolated nucleic acid molecule having a sequence of nucleotides or their chemical equivalents which directs a conformation defining a human neocentromere or a functional derivative thereof or a latent, synthetic or hybrid form thereof or its mammalian or non-mammalian homolog wherein the centromere associates with centromere binding proteins such as CENP-A and CENP-C but not limited to these proteins.

Reference herein to "latent" in relation to a centromere includes reference to a centromere not normally functional but nevertheless activatable under certain conditions. A latent centromere may also be considered as a neocentromere provided it has no substantial repetitive DNA sequences which are found on normal centromeres such as α-satellite DNA repeat sequences. A preferred repetitive DNA sequence in a non-human chromosome is considered to be a functionally equivalent repeat DNA to α-satellite DNA.

Accordingly, another aspect of the present invention contemplates the use of any method for identifying a neocentromere or a functional homolog, said method includes isolating DNA by chromatin immunoprecipitation using an antibody specific to mammalian CENP-A and/or CENP-C, or an antibody capable of cross interacting with mammalian CENP-A and/or CENP-C, amplifying the DNA isolated by immunoprecipitation and incorporating label into the amplified DNA and then using the amplified DNA to probe a DNA array comprising genomic DNA or its equivalent and identifying and isolating clones which hybridize to said immunoprecipitated DNA (52).

The size of the neocentromere in accordance with the present invention may range from about 50 bp to about 2000 kbp, from about 70 bp to about 1000 kbp, from about 75 bp to about 800 kpb, from about 80 bp to about 500 kpb, from about 85 bp to about 200 kpb, from about 90 bp to about 100 kbp, from about 100 bp to about 1 kpb, about 120 bp to about 500 bp, about 180 bp to about 300 bp. In one embodiment, the neocentromere is approximately 60-100 kbp. In another embodiment, the neocentromere is about 80 kpb. In a particularly preferred embodiment, the neocentromere is from about 50 kbp to about 2000 kpb. The neocentromere may encompass different structurally or functionally distinct domains such as CENP-A-binding or other centromere protein-binding domain, or domains showing different replication timing, chromatin structure, scaffold organisation, chemical modification status (e.g. acetylation, methylation, phosphorylation, poly-ADP-ribosylation). Provision is also given to the size of the neocentromere being larger than 2000 kbp to encompass all the functionally important domains.

The size of the mini-chromosome in accordance with the present invention may range from about 500 bp to about 20000 kbp, from about 700 bp to about 1000 kbp, from about 750 bp to about 8000 kpb, from about 800 bp to about 5000 kpb, from about 850 bp to about 2000 kpb, from about 900 bp to about 1000 kbp, from about 1000 bp to about 10 kpb, about 1200 bp to about 5000 bp, about 1800 bp to about 3000 bp. In one embodiment, the mini-chromosome is approximately 600-1000 kbp. In another embodiment, the mini-chromosome is about 800 kpb. In a particularly preferred embodiment, the neocentromere is from about 500 kbp to about 20000 kpb. The mini-chromosome may encompass different structurally or functionally distinct domains such as a neocentromere, replication origins, and telomeres. In a particularly preferred embodiment, the mini-chromosome contains a active neocentromere.

The nucleic acid molecule of the present invention may comprise a naturally occurring nucleotide sequence from a healthy human subject or may comprise the nucleotide sequence from a human subject exhibiting one or more chromosomal-dependent conditions such as a subject carrying mardel(10) chromosome or a chromosome conferring an equivalent or similar condition or may carry one or more nucleotide substitutions, deletions

and/or additions relative to the naturally or non-naturally occurring sequence. Such modifications are referred to herein as "derivatives" and include mutants, fragments, parts, homologs and analogs of the naturally occurring nucleotide sequence. Preferably, the derivatives of the present invention still define a functional neocentromere.

Reference herein to a "neocentromere" includes reference to a functional neocentromere or a functional derivative thereof meaning that it is capable of facilitating sister chromatid cohesion and chromosomal segregation during mitotic cell divisions and/or is capable of associating with CENP-A and/or CENP-C and/or other functionally important centromere proteins and/or is capable of interacting with anti-CENP-A antibodies or anti-CENP-C antibodies or antibodies to other functionally important centromere proteins. Generally, and preferably, the neocentromere is incapable of interacting with CENP-B or anti-CENP-B antibodies. Alternatively, the neocentromere may be a latent centromere capable of activation by epigenetic mechanisms or other relevant mechanisms. The neocentromere may also be a hybrid or other human, mammalian, plant or yeast neocentromeres. Synthetic neocentromeres provided by, for example, polymeric techniques to arrive at the correct conformation are also contemplated by the present invention. All such forms and definitions of neocentromeres are encompassed by use of this term.

Another aspect of the present invention provides an isolated nucleic acid molecule or chemical equivalent which comprises a nucleotide sequence or chemical equivalent directing a conformation which defines a neocentromere or a functional derivative thereof or a latent, synthetic or hybrid form thereof and wherein said neocentromere is substantially devoid of normal centromeric repetitive DNA such as α-satellite DNA and wherein the neocentromere is capable of associating with CENP-A or CENP-C or other functionally important centromere-binding proteins or antibodies thereto.

Preferably, the neocentromere is incapable of interacting with CENP-B or antibodies thereto.

In one embodiment, the neocentromere corresponds to a human genomic region which

maps between q24 and q26 on chromosome 10 and in particular q25 on chromosome 10.

The nucleic acid molecule or its chemical equivalent of the present invention defining a conformational neocentromere or functional derivative thereof or latent, synthetic or hybrid form thereof is useful *inter alia* for the generation of mini-chromosomes or artificial chromosomes such as human neocentromere-based mini-chromosomes (NC-MiCs), human artificial chromosomes (HACs), mammalian artificial chromosome (MACs), yeast artificial chromosomes (YACs) and plant artificial chromosomes (PLACs). Human NC-MiCs are particularly useful since they are capable of accommodating large amounts of DNA and are capable of propagation in human cells. The NC-MiCs are non-viral in origin and, hence, are more suitable for gene therapy by, for example, introducing therapeutic genes, than conventional viral based vector systems. Furthermore, the NC-MiCs remain extra-chromosomal and, hence, have no insertional/substitutional mutagenic potential. The essence of a NC-MiCs is the presence of a neocentromere or latent, synthetic or hybrid form thereof which enables stable segregation during cell division. The NC-MiCs also remain extra-chromosomal and, hence, are more suitable for gene therapy. Reference to "extra-chromosomal" means that it does not integrate into the main chromosome and, in effect, is episomal.

Accordingly, the present invention provides a genetic construct comprising an origin of replication for a eukaryotic cell and a nucleic acid molecule encompassing a eukaryotic neocentromere or a functional derivative thereof or a latent, synthetic, hybrid form thereof or its mammalian or non-mammalian homolog flanked by telomeric nucleotide sequences functional in the cell in which the genetic construct is to replicate and where said genetic construct when introduced into a cell is a replicating, extra chromosome element, either in a circular or linear form, which segregates with cell division.

More particularly, the present invention further contemplates a genetic construct in the form of a mini-chromosome or an artificial chromosome comprising an origin of replication for a mammalian, human, plant or yeast cell and a nucleic acid molecule encompassing a human neocentromere or a functional derivative thereof or a latent,

synthetic or hybrid form thereof or its mammalian or non-mammalian homolog flanked by telomeric nucleotide sequences or in a circular form carrying minimal or no telomere sequences functional in the cell in which the mini-chromosome or artificial chromosome is to replicate.

Another embodiment provides a genetic construct in the form of a mini-chromosome or an artificial chromosome comprising an origin of replication for a mammalian, human, plant or yeast cell and a nucleic acid molecule having a tertiary structure which defines a human neocentromere or a functional derivative thereof or a latent, synthetic or hybrid form thereof or its mammalian homolog flanked by telomeric sequences or in a circular form carrying minimal or no telomere sequences functional in the cell in which the mini-chromosome or artificial chromosome is to replicate.

Yet another embodiment is directed to a genetic construct in the form of a mini-chromosome or an artificial chromosome comprising an origin of replication for a mammalian, human, plant or yeast cell and a nucleic acid molecule having a sequence of nucleotides which directs a conformation defining a human neocentromere where the centromere associates with CENP-A and/or CENP-C and/or other centromere proteins or antibodies thereto and does not contain substantial α-satellite DNA repeat sequences, said nucleic acid molecule flanked by telomeric nucleotide sequences or in a circular form carrying minimal or no telomere sequences functional in the cell which the mini-chromosome or artificial chromosome is to replicate.

The present invention further provides a method for generating a mini-chromosome, said method comprising:-

introducing into a cell a truncation construct comprising a vector having telomeric sequences, a selectable marker, homologous targeting DNA on one or other of a q' or p' arm of the target chromosome;

selecting for cells expressing the selectable marker;

introducing into said cells a second truncation construct comprising the other of said q' or p' targeting DNA;

selecting for cells expressing the selectable marker associated with said second truncation construct; and

then isolating the truncated chromosome which comprises a neocentromere.

In one embodiment, the q' and p' targeting arms target the mardel(10) chromosome.

In a particularly preferred embodiment, the mini-chromosome is defined as NC-MiC1-5 as defined in Figure 1.

The present invention further provides a cell comprising a chromosome having q' and p' arms flanking a neocentromere wherein one or both q' and p' arms are truncated.

The above described method or its modified version thereof may also be used to introduce a gene or other nucleotide sequence such as for expression.

The present invention extends to eukaryotic cells such as human, primate, insect, yeast or other eukaryotic cells carrying the genetic constructs of the present invention and to proteins produced therefrom.

The genetic constructs of the present invention include mini-chromosomes and artificial chromosomes as well as DNA constructs useful in the generation of mini-chromosomes and artificial chromosomes.

The genetic constructs may also comprise marker genes, unique restriction sites, or recombination enhancing marker (e.g. LoxP DNA, for use with Cre recombinase) to facilitate insertion of adventitious DNA. Accordingly, the genetic constructs of the present

invention may further comprise adventitious or heterologous DNA encoding a product of interest. Preferred products of interest include pharmaceutically useful genes such as genes encoding cytokines, receptors, growth regulators and the like. Endogenous genes may also be replaced by wild-type genes or modified genes.

Specific DNA sequences intrinsically present on the genetic constructs (e.g. putative ESTs or expressed genes or unstable DNA elements) may also be identified, removed or modified by further genetic manipulation or engineering. Other DNA components (e.g. therapeutic or marker genes, LoxP DNA sequences, unique restriction sites such as I-SceI) may also be subsequently added to the genetic constructs to improve or broaden their utility or application.

The adventitious or heterologous DNA may also encode a molecule not synthesized in a sufficient amount in a particular subject and hence the increased copy number permits greater amounts of the molecule being synthesized.

Accordingly, the present invention contemplates a genetic construct comprising an origin of replication and a first nucleic acid molecule defining a human neocentromere or a functional derivative thereof or latent, synthetic or hybrid form thereof or a mammalian or non-mammalian homolog, a second nucleic acid molecule encoding a peptide, polypeptide or protein, wherein said first and second nucleic acid molecules are flanked by human telomeric sequences or in a circular form carrying minimal or no telomeric sequences functional in the cell in which the genetic construct is to replicate.

Reference herein to "segregate" preferably means mitotically stable segregation. Conveniently, stable segregation may be determined as the presence of a mini-chromosome or an artificial chromosome in more than 40-60% of daughter cells after 4-6 months of continuous passage.

The present invention extends to other mini-chromosomes or artificial chromosome analogs to the NC-MiCs described above such as HACs, MACs and PLACs, or similar

entities by any other descriptions or names such as micro-chromosomes, synthetic chromosomes, and variations thereof.

Another aspect of the present invention relates to peptides, polypeptides and proteins which bind, interact or otherwise associate with the human neocentromere of the present invention or its mammalian and non-mammalian homolog. Preferably, the molecules are proteins, referred to as primary (1°) proteins. The 1° proteins bind to the neocentromere and secondary (2°) proteins bind to the 1° proteins before or after association with the neocentromere. The identification of the human neocentromere in accordance with the present invention provides a mechanism for assaying 1° proteins and 2° proteins which may be important for screening chromosomes in, for example, genetic disorders. This is particularly the use in Down's Syndrome which results from defective chromosome segregation.

The 1° proteins are readily detected by, for example, a gel shift assay. The nucleic acid molecule of the present invention defining the human neocentromere is digested, labelled and contacted with nuclear extract putatively containing the 1° proteins and resolved on a gel. When a 1° protein binds to a fragment carrying a binding portion of the neocentromere, the DNA fragment migrates in the gel at a slower rate due to the bound protein.

The present invention extends to purified 1° proteins capable of association with the subject centromere and to genetic sequences encoding same and to antibodies thereto.

The neocentromeres of the present invention are readily identified and characterized using, for example, human fibrosarcoma cell lines. For example, DNA suspect of carrying a neocentromere is introduced into fibrosarcoma cells in a linear form generally together with a telomeric sequence. The cells are then screened for the presence of replicating, extra chromosomal and segregating elements, referred to as artificial chromosomes.

The present invention further encompasses eukaryotic cells carrying replicating, extra-

chromosomal and segregation nucleic acid molecules. Preferably, the eukaryotic cells are mammalian cells and most preferably human cells. The nucleic acid molecules according to this aspect of the present invention are preferably as herein described.

The following cell lines were deposited at the ECACC, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wilts, SP4 0JG, United Kingdom on 20 December 2000, under the listed Accession Numbers:-

Cell Line	Acronyn	Accession Number
CHO/BE ZB30	-	00122001
HT1080 MIC 1	NC-MiC1	00122002
HT1080-MIC 2	NC-MiC2	00122003
HT1080-MIC 3	NC-MiC3	00122004
HT1080-MIC 4	NC-MiC4	00122005
HT1080-MIC 5	NC-MiC5	00122006
HT1080-MIC 5a	NC-MiC5a	00122007
HT1080-MIC 5b	NC-MiC5b	00122008

The present invention is further described by the following non-liming Examples.

EXAMPLE 1

Cell culture and chemicals

BE2Cl-18-5f (abbreviated 5f) was cultured as previously described (27). HT1080 and derivatives were cultured in DMEM (Gibco BRL) with 10% v/v fetal calf serum (FCS). Hygromycin (Roche), Puromycin (Sigma corp), or Zeocin (Invitrogen) were added to medium at concentrations of 250 µg/ml, 1 µg/ml, or 200 µg/ml, respectively. Medium was supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco BRL). All cell lines were maintained at subconfluence. Microtubule depolymerizing agents colcemid (Gibco BRL) or nocadazole (Sigma) were added to medium at concentrations of 10 µM or 0.1 µg/ml for 1 or 6-12 hours, respectively, prior to cell harvesting. All chemicals used were of molecular biology grade and purchased from commercial sources.

EXAMPLE 2

Transfection of cell lines

Transfection of 5f and ZB30 cell lines were carried out using electroporation. Briefly, 10^7 log-phase cells were harvested, washed twice in PBS and resuspended in 800 µl electroporation buffer (20 mM Hepes, pH 7.0, 150 mM NaCl, 5mM KCl, 6 mM glucose, 2.5 mM NaOH, 1 mM Na_2HPO_4). 20 µg of linearized DNA was mixed with cells in a 0.4 cm cuvette and cells were electroporated at 1.2kV, 25uF using a Biorad Gene Pulser Electroporator. Transfection of HT1080 or derivative cells was carried out using either electroporation as previously described (30) or lipofection. For lipofection, the cells were plated at 1-3 x 10^5 cells/ml in 40 ml on a 150 mm plate one day prior to transfection. This resulted in 50-70% confluency on the day of the experiment. 2 ml of serum-free DMEM was used to dilute 100 µl Fugene 6 transfection reagent (Boehringer Mannheim) and the suspension was incubated at 37°C for 5 min. 20 µg of DNA was also diluted in 2 ml of serum-free DMEM. The diluted Fugene 6 was then added onto the DNA suspension drop-wise. The mixture was gently tapped and incubated for 15 min at room temperature. Following incubation, the DNA/Fugene 6 mixture was added onto the cells drop-wise. Cells were seeded into 96-well plates and selection was applied 24-48 hours post

transfection.

EXAMPLE 3

Microcell mediated chromosome transfer to HT1080

Microcell fusion was carried out as previously described (38). Log-phase donor ZB30 cells were incubated with colcemid (1 μg/ml) overnight. Cells were harvested after 48 hours and resuspended in percoll/serum-free DMEM (1:1) supplemented with 20 μg/ml of cytochalasin B (Sigma). The cell suspension was then subjected to centrifugation at 18,000 rpm for 90 min at 32°C in oakridge tubes (Nalgene). Both bands of cell-mix were pelleted and washed once with serum-free DMEM prior to filtration through isopore membranes of 30, 8 and 5 μM (Millipore Corp., MA). The microcells were then resuspended in serum-free DMEM containing 10 μg/ml PHAP (Difco, West Molesey, UK), and were allowed to agglutinate with the recipient HT1080 cells for 45min at 37°C. Following agglutination, cells were fused by addition of 50% w/v PEG (Boehringer Mannheim) and incubated for 2 min at room temperature followed by rinsing with serum-free DMEM. After incubation, cells were cultured overnight in DMEM containing 10% v/v FCS. The medium was then replaced with DMEM containing 200 μg/ml of zeocin and cells were maintained in selection for a period of 14 days before colonies were picked for further characterization.

EXAMPLE 4

FISH/Immunofluorescence

Combined FISH/Immunofluorescence was carried out using a modified procedure previously described (25,51). FISH using pan-α-satellite probe pTRA7 and PNA-FISH of telomeric sequences (Perspectives Biosystem, MA) were carried out as previously described (39,40). Epifluorescence microscopy was performed on a Zeiss Axoplan II (Carl Zeiss, Carnegie, Australia) mounted with appropriate filter sets. Images were digitally acquired using a cooled charged-coupled device video camera (SenSys 2, Photometrics, Tuscon, AZ, USA) connected to a PowerMac G3 personal computer controlled by the software IP Lab Version 2.5.5 (Scanalytics Inc., Fairfax, VA,USA). Chromosome painting

experiments were carried out using WCP Chromosome Paint Kit (Vysis Inc.) according to the manufacturer's instructions. Subchromosome-10 paints were derived from somatic cell radiation hybrid genomic DNA obtained from M. Rocchi (University of Bari). Inter-Alu amplification of somatic cell hybrid DNA was carried out using primers 5' GGATTACAGGYRTGAGCCA [SEQ ID NO:1] and 5' RCCAYTGCACTCCAGCCTG [SEQ ID NO:2] as previously described (41). Labelling of paint-probes and FISH were carried out using standard techniques.

Polyclonal anti-CENP-A, monoclonal anti-CENP-B, polyclonal anti-CENP-C, and CREST-6 antisera have been previously described (42,43,44,27). Polyclonal anti-CENP-E (45), anti-CENP-F (46), and anti-hBUB1 (47) were provided by T.J. Yen (Fox Chase Cancer Center), polyclonal anti-hZW10 (48) by B. Williams and M.L Goldberg (Cornell University), polyclonal p55CDC (49) by J. Weinstein (Amgen Corp) and polyclonal anti-TRF1 (32) by Titia de Lange (Rockefeller University, NY). Antisera CREST-6 was from a patient with the autoimmune CREST disease, containing antibodies against centromere components including CENP-A and CENP-B (51). Other secondary antibodies were purchased from the Jackson ImmunoResearch Laboratory Inc. (West Grove, Pennsylvania, USA).

EXAMPLE 5

Truncation constructs

Truncation constructs contained either pGK:hygromycin, pGK:puromycin, or pGK:neomycin resistance gene cassettes. A 2kb array of human telomeric repeats was obtained from pBS Sal-tel(5) plasmid (28,50). Genomic cosmid clones containing DNA corresponding to the p' and q' arms of mardel(10) have been previously described. 5-10 kb fragments lacking high-copy repeat DNA sequences (as evidenced by a lack of COT-1 hybridization following Southern hybridization), were subcloned in both orientations into truncation vectors using standard techniques. Alternatively genomic DNA was PCR amplified directly from BAC clones using Long Range PCR kit (Boehringer Mannheim) for cloning into truncation vectors. All truncation constructs were made in pAlter

(Promega Corp.) vector backbone.

EXAMPLE 6

Concatamerization of zeocin resistance marker

A zeocin resistance cassette from the pZeoSV2(+) plasmid (Invitrogen) was PCR amplified with the addition of flanking *Not*I restriction sites. This was cloned into pGEM-T (Promega Corp.). 100 µg of this plasmid was digested with *Not*I, and purified by phenol/chloroform extraction. To produce concatamers, 10 µl ligase buffer, 40 units of T4 ligase, and 5 µl of 100mM ATP were added to the digested DNA in a total volume of 100 µl. The ligation reaction was carried out overnight and 1 µl of the completed reaction was tested by pulsed field gel electrophoresis to determine the size of the resulting concatamers. DNA was phenol/chloroform-extracted and ethanol-precipitated prior to transfection.

EXAMPLE 7

p' arm truncation screening using dot blot hybridization

Cells in 24-well plates were harvested by trypsinization and transferred directly to a Minifold dot blotting apparatus (96 per blot; Schleriche and Schuell Inc.) containing Hybond N+ (Amersham Corp.) under vacuum. Following transfer, Hybond N+ was denatured for 10 min (1M NaOH, 1M NaCl), neutralized twice for 5 min (1 M Tris-HCl, 1.5 M NaCl) followed by a final wash in 2X SSC. Membranes were then baked for 1.5 hours at 80°C and were probed using ^{32}P-labelled pAlter vector (Promega Corp.) DNA using standard techniques.

EXAMPLE 8

Mardel(10) tagging and transfer into HT1080 cells

A CHO-based somatic cell hybrid line (designated 5f) containing the mardel(10) chromosome (27) was used. Initial telomere-associated chromosome truncation (TACT)

using a system similar to that previously described (9,12,28) was performed on 5f employing the truncation vectors shown in Figure 1A (described below). An exhaustive screening of over 25,000 drug-resistant colonies transfected with different truncation vectors yielded no positive truncation events, suggesting that the 5f cell line was not a suitable host for TACT. The inventors then decided to transfer the mardel(10) chromosome into human HT1080 cells since this cell line is known to be homologous recombination proficient (29,30), shows telomerase activity (31,32,33), and is a good recipient for microcell-mediated chromosome transfer (34,35). A random insertion approach was first used to tag mardel(10) in 5f cells with a zeocin resistance gene cloned in pGEM-T vector. In order to facilitate subsequent FISH screening for chromosome tagging, the zeo^R/pGEM-T construct containing zeocin resistance gene was concatamerized and only DNA fragments larger than 50 kb were used to transfect 5f cells. Screening 63 individual zeocin-resistant colonies identified a single cell line (designated ZB30) in which mardel(10) was tagged at its distal q' region (Figure 2A). This cell line was used as a donor in microcell-mediated chromosome transfer into HT1080 cells. 15 out of 60 zeocin-resistant microcell-fusion colonies were shown to contain mardel(10). Extensive FISH and immunofluorescence analyses of these cell lines using BACs from the neocentromere region, pan-α-satellite, CHO genomic DNA, and a host of anti-centromere antibodies confirmed that the mardel(10) chromosome and its neocentromere were intact, and that no integration of any α-satellite repeats or CHO DNA had occurred on the chromosome. One of these cell lines, designated ZBHT-14, was employed in subsequent truncation experiments.

In addition to cell lines such as ZBHT-14 which have acquired an intact mardel(10), a number of lines containing randomly truncated derivatives of this chromosome were also detected in the fusion products; the generation and transfer of such broken chromosomal fragments appeared to be a relatively common occurrence associated with the microcell fusion procedure (36). One cell line (designated NC-MiC1) was of interest because it carried a mini-chromosome that was shown by FISH to contain approximately 2 Mb of DNA around the previously mapped 10q25 neocentromere domain (27) (Figure 1C). This cell line was retained for further characterization and truncation studies.

EXAMPLE 9

Truncation constructs

A complete physical map was prepared containing over 50 BAC and cosmid clones covering approximately 3 Mb of the 10q25 neocentromere region (Figure 1C). Based on this map, a number of truncation constructs containing different targeting DNA from this region were designed. These constructs contained either hygromycin (for q'-arm truncation), neomycin or puromycin (for p'-arm truncation) resistance genes, terminal cloned human telomere sequences, and 5-10 kb of targeting DNA from either the q' or p' arm of mardel(10) (Figure 1A). Initial TACT experiments were performed on the q' arm, followed by truncation of the p' arm (Figure 1B).

EXAMPLE 10

Truncation of q' arm

ZBHT-14 and NC-MiC1 cell lines were used in q' truncation experiments. Both cell lines were transfected with a hyg^R (hygromycin resistance) truncation-construct containing a 6-kb targeting DNA derived from the Y3C94 cosmid (which overlaps with BAC B79E16; Figure 1C). This targeting site is situated approximately 0.2 Mb from the anti-centromere antibody binding NC domain (27).

For the ZBHT-14 cell line, initial screening for q' truncation of mardel(10) was performed by plating the resulting hygromycin-resistant colonies in zeocin. This allowed the identification of cell lines which have lost the zeocin-containing chromosome portion of mardel(10) and were, therefore, zeocin sensitive. From a total of 7,300 hygromycin-resistant colonies, 210 were shown to be zeocin sensitive. FISH analysis revealed that most of these colonies contained random truncations or other unknown rearrangements. One cell line (designated NC-MiC2) appeared to have undergone the desired targeted truncation and was characterized further.

FISH analysis of NC-MiC2 using a host of known cosmid or BAC clones mapped along the 10q25 neocentromere region revealed that clones on or proximal to the targeting DNA were present (e.g. Y3C94; Figure 2B), whereas all clones distal to the targeting DNA were absent (e.g. Y3C109; Figure 2C) (summarized in Figure 1C). To more closely determine the site of truncation, three ~10-kb PCR fragments (F1-F3) immediately adjacent to either side of the intended targeting site (Figure 1D) were prepared and used in FISH analysis. The results indicated that only the fragment (F1) located proximally to the targeting DNA gave a positive signal (Figure 2D), whilst the two distal fragments F2 and F3 were both negative (Figure 2E). Further FISH using TTAGGG telomere-repeat peptide nucleic acid (PNA) probe demonstrated seeding of telomeric sequences on the truncated chromosome (Figure 2H). Low-stringency FISH using pan-α-satellite probe and immunofluorescence using anti-CENP-B antibody (Figure 2F) confirmed that no α-satellite DNA has been inserted into the truncated chromosome. These data strongly support a targeted-truncation event leading to the removal of most of the q' arm of mardel(10) in the production of NC-MiC2.

Mitotic stability of NC-MiC2 was determined by comparing cells grown in the presence or absence of hygromycin over 20 cell divisions. A similar retention rate of 85% was observed for cells grown with or without drug selection, suggesting that NC-MiC2 was mitotically stable. To further investigate whether this chromosome maintained a functional neocentromere, immunofluorescence studies were carried out using CREST-6 autoimmune serum (27) and specific antibodies to the histone H3-like protein CENP-A, CENP-C, and the kinesin-like motor protein, CENP-E. Strong antisera signals that co-localized with the E8 BAC previously mapped to the neocentromere (NC) domain (37) were observed for all the proteins tested (e.g. Figure 2G) thereby demonstrating neocentromere function on NC-MiC2.

Transfection of the NC-MiC1 cell line with the Y3C94 DNA-containing q' truncation construct resulted in over 1000 hygromycin-resistant colonies. These were screened for the loss of the vector DNA contained in the construct (see Figure 1A and Example 7) that was indicative of a targeted truncation event. Detailed FISH analysis of one resulting cell line,

NC-MiC3 with q' cosmids and BACs, and the PCR probes F1-F3, demonstrated a correctly targeted truncation at the intended Y3C94 site (examples of FISH results are shown in Figures 3A-B, and summarized in Figure 1C). Further p' mapping and analysis of NC-MiC3 are described below.

EXAMPLE 11

Truncation of p' arm

NC-MiC2 was subjected to further truncation using constructs carrying puromycin or neomycin markers and targeting DNA from 3 different p' regions (Figure 1C). Puromycin- or neomycin-resistant colonies, generated through several independent transfection experiments, were screened for possible targeting by probing for loss of vector DNA as described above, as well as by dual-color FISH using E8 BAC and a distal p'-arm BAC (B10K1) (see Figure 1B) to identify truncated NC-MiC2 derivatives. This analysis indicated that most colonies did not appear to contain the intended targeted chromosomal truncations of NC-MiC2. However, two cell lines (NC-MiC4 and NC-MiC5) were identified that showed truncation at p' sites relatively close to the neocentromere antigen-binding region (27). These were characterized in greater detail.

EXAMPLE 12

Structure of NC-MiCs 3, 4 and 5

Figure 1C summarizes the results for detailed FISH mapping of NC-MiCs 3, 4 and 5 using probes of known map position (some examples are shown in Figures 3-5). All three NC-MiCs showed the expected q' truncation within Y3C94. On the p' arm, chromosome truncation was seen between BACs Y13C12(present)/B179N3(absent), Y13C12 (present)/B43A11(absent), and BA48L24(present)/BA69K10(absent), for NC-MiC3 (and its predecessor NC-MiC1), NC-MiC4, and NC-MiC5, respectively (Figures 3C/D, 4A/B, and 5A/B). The intensity of the positive FISH signals on the different NC-MiCs were indistinguishable from those seen on the normal chromosomes 10 in HT1080 cells for all the cosmid and BAC probes tested, suggesting that no duplication of DNA has occurred

during formation of the NC-MiCs. Low-stringency FISH hybridization using pan-α-satellite DNA probe (Figures 3E, 4C and 5C), and immunofluorescene using anti-CENP-B antibody (Figures 4E and 5D) demonstrated the absence of centromeric α-satellite DNA. When the different NC-MiC cell lines were analyzed by FISH using whole-chromosome paints for all 24 human chromosomes, only the chromosome 10-paint produced positive signals on the NC-MiCs, suggesting that no detectable amount of genomic DNA from other chromosomes have been translocated onto the NC-MiCs during their formation (Figure 6A). Further analysis using a number of subchromosome 10-paints (see Figure 1B for locations) similarly demonstrated the absence of DNA from non-10q25 regions on the NC-MiCs (Figure 6B). It can, therefore, be concluded that NC-MiCs 3, 4 and 5 each contain single-copy DNA derived solely from the 10q25 neocentromere region, with total sizes estimated of approximately 1.6, 1.6 and 0.8 Mb, respectively (Figure 1C).

High-molecular weight genomic DNA was prepared from the different cell lines and subjected to pulsed field gel electrophoresis under varying conditions that resolved DNA of up to 6 Mb. Only the NC-MiC3 migrated into the gel, suggesting that this is a linear mini-chromosome and raising the possibility that the other NC-MiCs are circular structures. Comparison with yeast chromosome markers indicated a size of 1.6 Mb on the PFGE gel for NC-MiC3. FISH using a pan-telomere probe or immunofluorescence using an antibody to the telomere repeat-binding factor TRF1 produced signals on telomeric ends of all normal chromosomes in HT1080 cells but not on any of the NC-MiCs, including the linear NC-MiC3 (e.g. Figure 4D). This could be due to inability of the technique to detect low levels of telomere sequences and/or the circular nature of the NC-MiCs.

EXAMPLE 13

NC-MiC stability and neocentromere activity

The mitotic stability of NC-MiCs 3, 4 and 5 was assayed following ≥20 cell divisions in culture media with and without selection. BAC E8 was used in FISH experiments to identify the NC-MiCs and 100 cells were scored for each cell line. For both NC-MiC3 and NC-MiC4, similar retention rates of approximately 80% were observed in the presence or

absence of drug selection, suggesting that both chromosome derivatives were stable even in the absence of selection pressure. A retention rate of 36% was initially observed for the NC-MiC5 cell line with selection. Following removal of selection and culturing for 20 divisions, 37% of cells retained NC-MiC5, again suggesting mitotic stability in the absence of selective pressure; the reduced retention rate of NC-MiC5 compared to NC-MiCs 3 and 4 was most likely related to inherent genomic instability seen in this particular cell line.

As NC-MiC5 appeared to be the smallest derivative constructed, and because of the observed background genome instability in the original cell line, the inventors subcloned this line and examined the stability of NC-MiC5 in resulting clones. Two of the subclones (NC-MiC5a and NC-MiC5b) exhibited greatly increased stability of the NC-MiC5. Following ≥50 cell divisions in the absence of drug selection, these two cell lines demonstrated retention rates of 90% and 93%, respectively. These retention rates were not significantly different from those (90% and 91%, respectively) seen in cells passaged for the same number of divisions in the presence of selection. NC-MiC5a cells contained either one copy (76% of cells) or two copies (14% of cells) of the mini-chromosome, while only one copy was consistently detected in the NC-MiC5b cells. The structures of the NC-MiC5a and 5b were shown by detailed FISH analysis to be identical to the original NC-MiC5 chromosome (Figures 1C and 5).

Immunofluorescence detection was used to investigate the functional status of the neocentromeres on the NC-MiCs. Antisera to a host of centromere-associated proteins, including CENP-A, CENP-B, CENP-C, CENP-E, CENP-F, hZW10, p55CDC, and BUB1 were tested. All proteins with the exception of CENP-B were clearly detected on each of the NC-MiCs (some examples are shown in Figures 3F-G, 4F-H, 5E-G). These protein-distribution profiles were indistinguishable from those previously established in the parental mardel(10) chromosome (25,27), confirming that the NC-MiC derivatives contained full neocentromere activity.

EXAMPLE 14

Cell culture and transfection of NC-MiC2

NC-MiC2 was cultured in DMEM (Gibco BRL) with 10% v/v FCS. Hygromycin (Roche) was added to medium at a concentration of 250 μg/ml. Transfection of NC-MiC2 was carried out using electroporation or lipofection. Electroporation was performed (0.4 kV, 250 uF) using a Biorad Gene Pulser Electroporator. For lipofection, the cells were plated one day prior to transfection to give 60-70% confluency at the time of transfection. Two ml of diluted Fugene 6 transfection reagent (100 μl in a total of 2 ml containing 20 μg DNA) (Boehringer Mannheim) was added onto cells drop-wise. The DNA used in transfection was a TACT construct containing human telomeric sequence htel, two loxP sites flanking a neomycin resistance gene, targeting genomic DNA and a blasticidin resistance gene (Figure 7A). The antibiotic selection was applied 24-48 hours post-transfection at the concentration of 250 μg/ml for a period of 14 days before the colonies were picked for further characterization.

EXAMPLE 15

Truncation of p' arm of NC-MiC2

TACT experiments were performed on the p' arm in NC-MiC2 cells using a neomycin-resistance truncation-construct containing a 4 kb targeting DNA derived from the Y13C15 cosmid/B137il BAC, two loxP sites, neomycin resistance gene and a blasticidin resistance gene outside the targeting DNA. A successful targeting event would result in the loss of blasticidin resistance gene. Of 10,000 neomycin-resistant cell lines, approximately 10% were blasticidin sensitive. Sensitivity to blasticidin was determined by culturing the clones in 5 μg/ml of blasticidin. Clones that were neomycin resistant but blasticidin sensitive were subjected to FISH analysis.

EXAMPLE 16

Characterization of NC-MiC6

One cell line (NC-MiC6) appeared to have undergone targeted truncation and was characterized extensively by FISH. Cosmids and BACs proximal to the targeting site were found to be present, whereas all clones distal to this site were absent (summarized in Figure 7C). Based on the inventors' FISH and recently available genome sequence data, the inventors estimated the size of NC-MiC6 to be 1.2 Mb.

No α-satellite (pTRA7) and CENP-B binding were detected on the truncated chromosome. Immunofluorescence using CREST-6 autoimmune serum confirmed neocentromere activity on NC-MiC6. These data support, therefore, a targeted-truncation event that removed most of the p' arm of mardel(10) in NC-MiC2. The mitotic stability of NC-MiC6 was assayed with and without selection for up to 60 divisions in culture. Retention rates of >80% were observed after 20 cell divisions, 75% at 40 cell divisions and with 70% ag 60 divisions (Figure 8) in the absence of selection. In the presence of neomycin, 85% was maintained after 40 divisions and as high as 75% of NC-MiC6 was retained over 60 cell divisions, with loss rate of 0.42% per division, suggesting that NC-MiC6 was mitotically stable over time.

The presence of two loxP sites may be used for excision of neomycin resistance gene and insertion of new gene into specific site using cre-recombinase. The excision and insertion of genes is carried out either *via* transient transfection of plasmid containing the cre-recombinase gene or protein transfection of cre-recombinase.

EXAMPLE 17

Microcell mediated chromosome transfer to mouse embryonic stem ES cells expressing GFP (green fluorescent protein) and to mouse F9 teratocarcinoma cells

Mouse embryonic stem cells were cultured in ES medium (Gibco BRL) with 20% v/v FCS. Mouse F9 cells were cultured in DMEM (Trace Biosciences) with 10% v/v FCS.

Transfection of ES cells was carried out using electroporation at 0.8 kV and 3 uF using a Biorad Gene Pulser Electroporator. The DNA used in transfection was pEGRP-N1 (Clontech) containing the gene encoding the green fluorescent protein and a neomycin resistance gene. Antibiotic selection was applied 24-48 hours post transfection (250 μg/ml G418 neomycin) for a period of 14 days before the colonies were picked and scaled up for screening.

Microcell fusion was carried out as previously described. Log-phase donor ZB30 cells arrested in colcemid for 48 hours were resuspended in percoll/serum-free DMEM (1:1) supplemented with 20 μg/ml of cytochalasin B (Sigma). The cell suspension was then subjected to centrifugation at 18,000 rpm for 90 min at 32°C. Both bands of cell-mix were pelleted, washed with serum-free DMEM and filtered through isopore membranes of 30, 8 and 5 μM (Millipore Corp., MA). Microcells were then fused with recipient neomycin-resistant ES-GFP or F9 cells by addition of 50% w/v PEG (Roche) for 2 min at room temperature. After incubation, cells were rinsed and cultured overnight in ES media containing 20% v/v FCS or DMEM containing 10% v/v FCS followed by addition of antibiotic selection (250 μg/ml G418 neomycin and 100 μg/ml of zeocin for ES cells and 100 μg/ml zeocin for F9 cells) 24 hrs later.

EXAMPLE 18

Characterization of ES-GFPmar (10)#1 and F9-4-5mar(10) cell lines

Extensive FISH analysis of ES-GFPmar(10)#1 and F9-4-5mar(1) demonstrated that the mardel(10) chromosome and the neocentromere contained therein were intact. No mouse centromeric/pericentromeric major and minor satellite DNA or genomic DNA was detected on the marker chromosome in either cell line. In addition, mardel(10) was the only human chromosome present in these cell lines. The stability of mardel(10) was assayed with selection for up to 45 divisions in culture and the marker chromosome showed mitotic stability in both cell lines.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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CLAIMS

1. An isolated nucleic acid molecule comprising a sequence of nucleotides derived from a eukaryotic chromosome and encompassing a neocentromere which nucleic acid molecule exists in a truncated or modified form in a compatible cell or, when introduced by transfection as a pre-fabricated DNA entity into a compatible cell, is capable of replicating, acting as an extra chromosomal element and segregating with cell division.

2. An isolated nucleic acid molecule of Claim 1 wherein the eukaryotic chromosome is derived from a mammal including a human or primate or plant, avian species, insect, worm, fungus, yeast or reptile.

3. An isolated nucleic acid molecule of Claim 2 wherein the eukaryotic chromosome is derived from a human.

4. An isolated nucleic acid molecule of Claim 2 wherein the eukaryotic chromosome is derived from a livestock animal.

5. An isolated nucleic acid molecule of Claim 1 wherein the molecule is generated by a combination of targeted telomere-associated truncation of a q' arm and a random truncation of a p' arm of the mardel(10) chromosome or its equivalent.

6. An isolated nucleic acid molecule of Claim 5 wherein said nucleic acid molecule comprises a neocentromere at a location equivalent to between q24 and q26 on chromosome 10 or its equivalent.

7. An isolated nucleic acid molecule of Claim 6 wherein said nucleic acid molecule comprises a neocentromere at a location equivalent to at or about q25 on chromosome 10 or its equivalent.

8. An isolated nucleic acid molecule of Claim 7 wherein said nucleic acid molecule comprises a neocentromere at a location equivalent to at or about q25.2 on chromosome 10 or its equivalent.

9. A nucleic acid molecule of Claim 1 or 6 or 7 or 8 wherein the region corresponding to a neocentromere is substantially devoid of α-satellite DNA.

10. A nucleic acid molecule of Claim 9 wherein the nucleic acid molecule is from about 0.5 to about 2.0 Mb in size.

11. A nucleic acid molecule of Claim 10 wherein the nucleic acid molecule is from about 0.8 to about 1.6 Mb in size.

12. A method for identifying a neocentromere or a functional homolog, said method comprising isolating DNA by chromatin immunoprecipitation using an antibody specific to mammalian CENP-A and/or CENP-C or an antibody capable of cross interacting with mammalian CENP-A and/or CENP-C, amplifying the DNA isolated by immunoprecipitation and incorporating label into the amplified DNA and then using the amplified DNA to probe a DNA array comprising genomic DNA or its equivalent and identifying and isolating clones which hybridize to said immunoprecipitated DNA.

13. A method of Claim 12 wherein the mammal is a human, livestock animal, companion animal or laboratory test animal.

14. A method of Claim 13 wherein the mammal is a human.

15. A method of Claim 14 wherein the neocentromere is at a location equivalent to between p24 and p26 on chromosome 10 or its equivalent.

16. A method of Claim 15 wherein the neocentromere is at a location at or about q25 on chromosome 10 or its equivalent.

17. A method of Claim 16 wherein the neocentromere is at a location at or about q25.2 on chromosome 10 or its equivalent.

18. An isolated nucleic acid molecule in the form of a human neocentromere-based mini-chromosome (NC-MiCs), said NC-MiCs comprising a neocentromere or a latent, synthetic or hybrid form thereof which enable stable segregation during cell division.

19. An isolated nucleic acid molecule of Claim 18 generated by a combination of targeted telomere-associated truncation of a q' arm and a random truncation of a p' arm of the mardel(10) chromosome or its equivalent.

20. An isolated nucleic acid molecule of Claim 19 wherein the neocentromere is at a location equivalent to between q24 and q26 on chromosome 10 or its equivalent.

21. An isolated nucleic acid molecule of Claim 20 wherein the neocentromere is at a location at or about q25 on chromosome 10 or its equivalent.

22. An isolated nucleic acid molecule of Claim 20 wherein the neocentromere is at a location at or about q25.2 on chromosome 10 or its equivalent.

23. A method for generating a mini-chromosome, said method comprising:-

introducing into a human or mammalian cell which carries a chromosome containing a neocentromere, a truncation construct comprising a vector having telomeric sequences, a selectable marker, homologous targeting DNA on one or other of the q' or p' arm of the target chromosome;

selecting for cells expressing the selectable marker;

introducing into said cells a second truncation construct comprising the other of said q' or p' targeting DNA;

selecting for cells expressing the selectable marker associated with said second truncation construct; and

then isolating the truncated chromosome which comprises a neocentromere.

24. A method of Claim 23 wherein the cell is a human cell.

25. A method of Claim 24 wherein the neocentromere is at a location equivalent to between q24 and q26 on chromosome 10 or its equivalent.

26. A method of Claim 24 wherein the neocentromere is at a location at or about q25 on chromosome 10 or its equivalent.

27. A method of Claim 24 wherein the neocentromere is at a location at or about q25.2 on chromosome 10 or its equivalent.

28. An isolated cell comprising a chromosome having q' and p' arms flanking a neocentromere wherein one or both q' and/or p' arms are truncated.

29. An isolated cell line deposited at ECAAC under Accession 00122001 (CHO/BE ZB30).

30. An isolated cell line deposited at ECAAC under Accession 00122002 (HT1080- MIC 1).

31. An isolated cell line deposited at ECAAC under Accession 00122003 (HT1080- MIC 2).

32. An isolated cell line deposited at ECAAC under Accession 00122004 (HT1080- MIC 3).

33. An isolated cell line deposited at ECAAC under Accession 00122005 (HT1080- MIC 4).

34. An isolated cell line deposited at ECAAC under Accession 00122006 (HT1080- MIC 5).

35. An isolated cell line deposited at ECAAC under Accession 00122007 (HT1080- MIC 5a).

36. An isolated cell line deposited at ECAAC under Accession 00122008 (HT1080- MIC 5b).

37. Use of an isolated nucleic acid molecule according to Claim 1 in the manufacture of a mini-chromosome for use in gene therapy.

38. Use of Claim 37 wherein the mini-chromosome is an NC-MiC.

39. Use of Claim 37 wherein the mini-chromosome is a YAC.

40. Use of Claim 37 wherein the mini-chromosome is a HAC.

41. Use of Claim 37 wherein the mini-chromosome is a MAC.

42. Use of Claim 37 wherein the mini-chromosome is a PLAC.

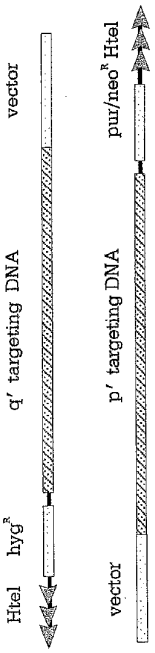
HtelI
hygR
q′ targeting DNA
vector
vector
p′ targeting DNA
pur/neoR HtelI

Figure 1A


chromosome 10
mardel(10)
NC-HAC2
NC-HAC3, 4, 5
164
174
174
172
85
174
172
p
q
zeo^R
q' (42Mb)
p' (16Mb)
Tc1
B10K1
Tc2

Figure 1B

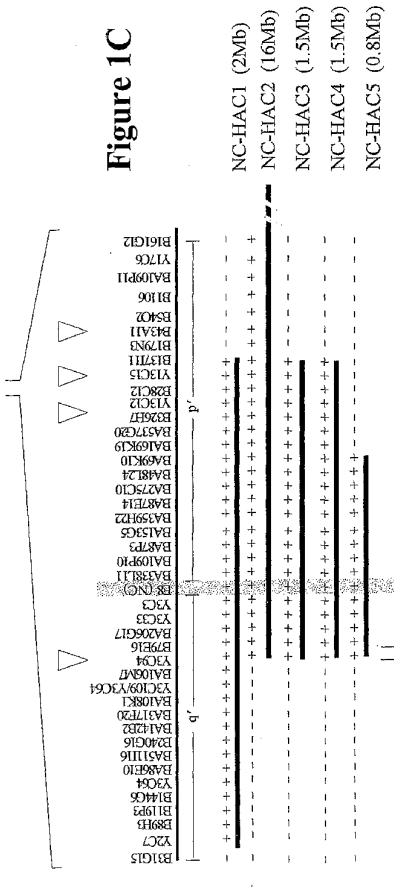
Figure 1C
NC-HAC1 (2Mb)
NC-HAC2 (16Mb)
NC-HAC3 (1.5Mb)
NC-HAC4 (1.5Mb)
NC-HAC5 (0.8Mb)
p'
q'

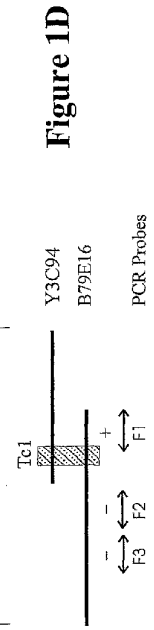
Figure 1D
Y3C94
B79E16
PCR Probes
Tc1
F1
F2
F3

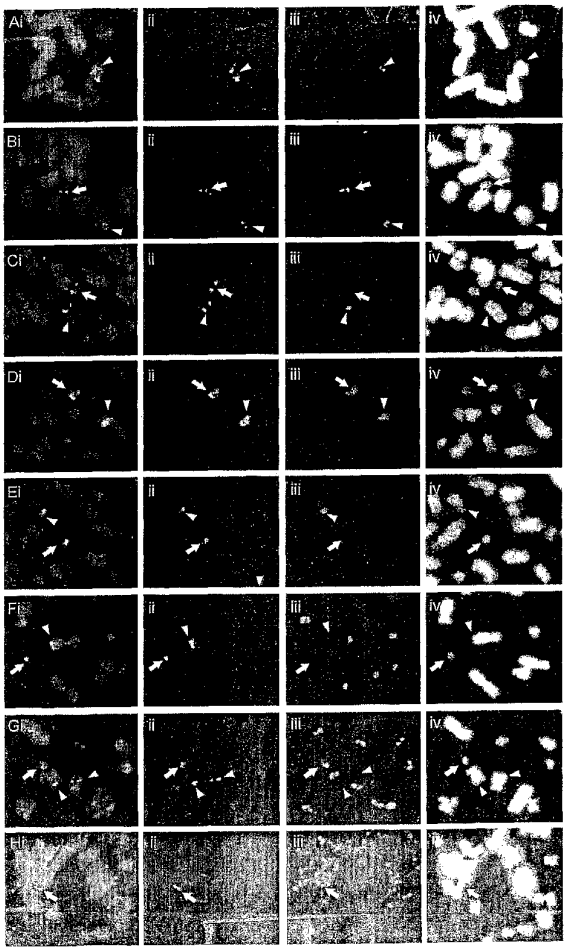
Ai
ii
iii
iv
Bi
ii
iii
iv
Ci
ii
iii
iv
Di
ii
iii
iv
Ei
ii
iii
iv
Fi
ii
iii
iv
Gi
ii
iii
iv
Hi
ii
iii
iv

Figure 2

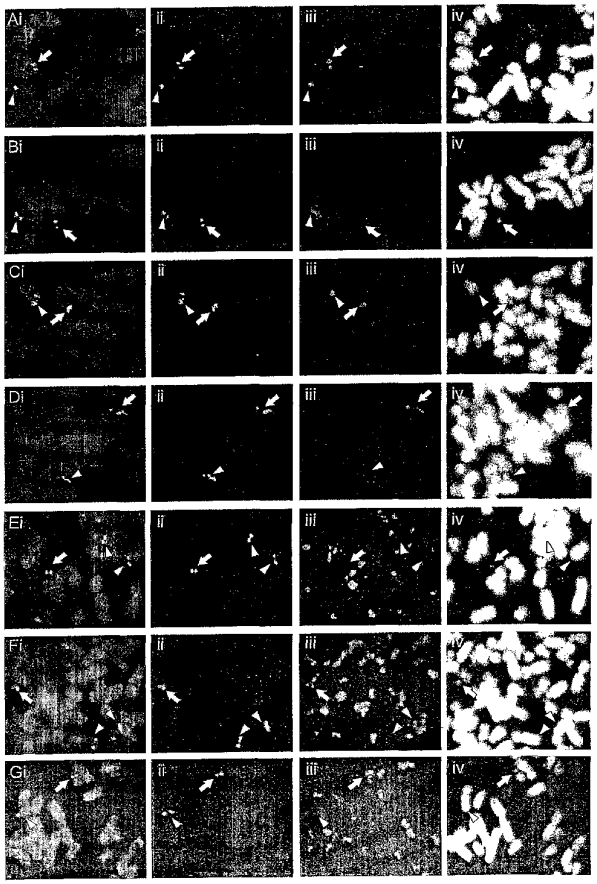
Ai
ii
iii
iv
Bi
ii
iii
iv
Ci
ii
iii
iv
Di
ii
iii
iv
Ei
ii
iii
iv
Fi
ii
iii
iv
Gi
ii
iii
iv

Figure 3

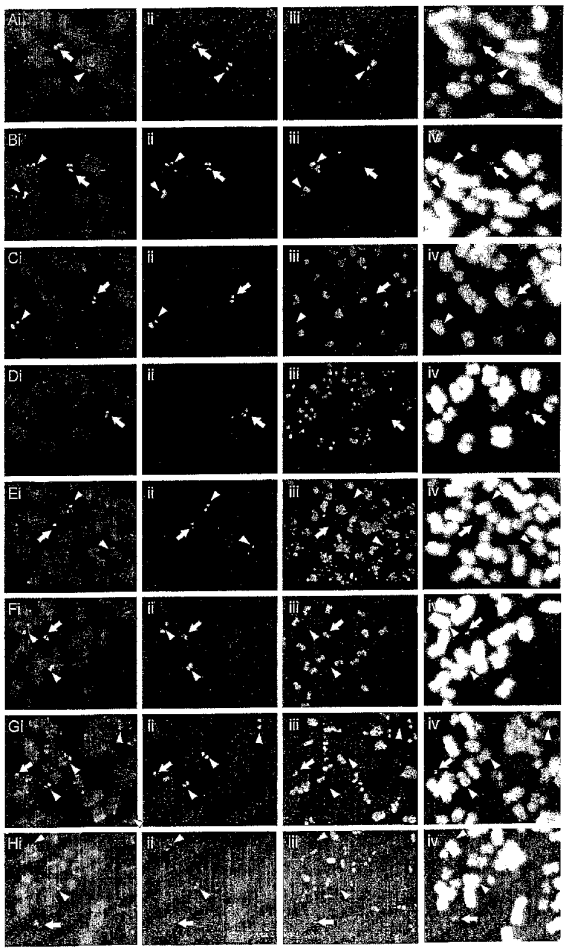
Ai
ii
iii
iv
Bi
ii
iii
iv
Ci
ii
iii
iv
Di
ii
iii
iv
Ei
ii
iii
iv
Fi
ii
iii
iv
Gi
ii
iii
iv
Hi
ii
iii
iv

Figure 4

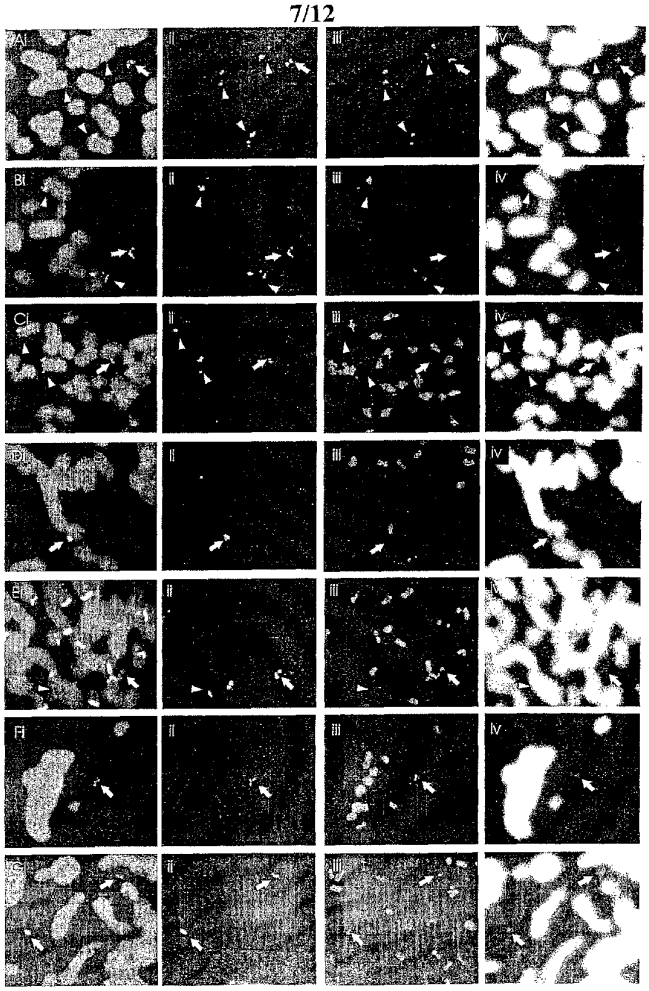
Ai
ii
iii
iv
Bi
ii
iii
iv
Ci
ii
iii
iv
Di
ii
iii
iv
Ei
ii
iii
Fi
ii
iii
iv
Gi
ii
iii

Figure 5

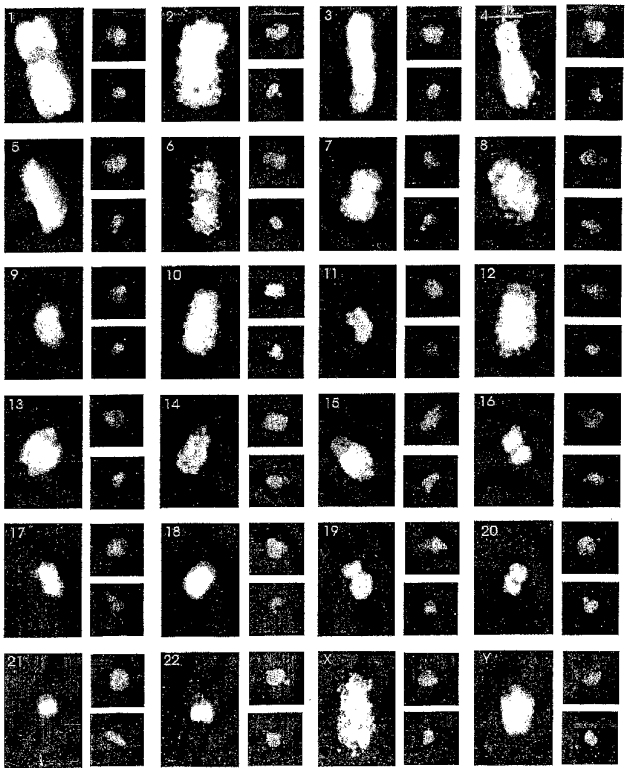

Figure 6A

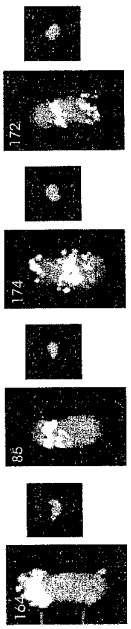

Figure 6B

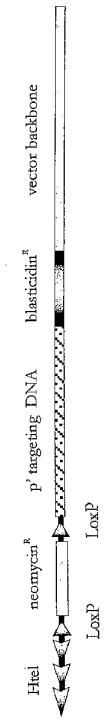

Figure 7A

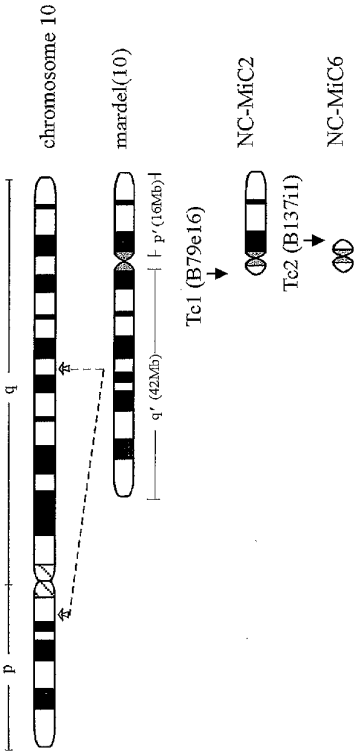
chromosome 10
mardel(10)
NC-MiC2
NC-MiC6
p
q
q' (42Mb)
p' (16Mb)
Tc1 (B79e16)
Tc2 (B137i1)

Figure 7B

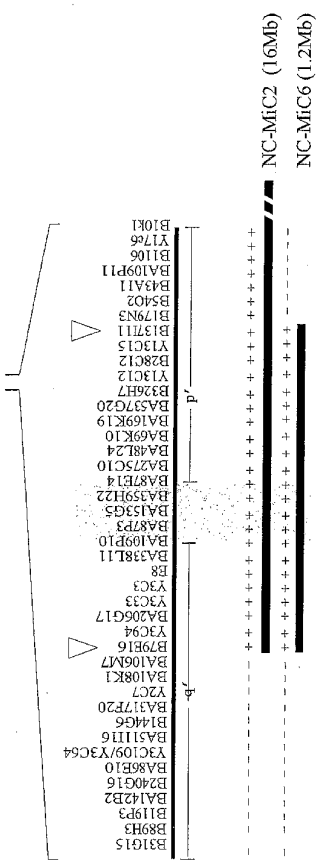
NC-MiC2 (16Mb)
NC-MiC6 (1.2Mb)
p'
q'
B10K1
Y17e6
B1106
BA109P11
B43A11
B540Z
B179N3
B137I11
Y13C15
B28C12
Y13C12
B326H7
BA537G20
BA169K19
BA69K10
BA48L24
BA275C10
BA87E14
BA359H22
BA153G5
BA87P3
BA109P10
BA338L11
E8
Y3C3
Y3C33
BA206G17
Y3C94
B79E16
BA106M7
BA108K1
Y2C7
BA317P20
B144G6
BA5111I6
Y3C109/Y3C64
BA86B10
B240G16
BA142B2
B119P3
B89H3
B316I15

Figure 7C

cell line	passage no	division no	Drug selection	No of cells scored	percentages of retention	loss per division
MIC-6	5	10	Neomycin	20	19/20 (95%)	0.50%
			None	20	18/20 (90%)	1.00%
	10	20	Neomycin	20	17/20 (85%)	0.75%
			None	20	16/20 (80%)	1.0%
	20	40	Neomycin	20	16/20 (80%)	0.5%
			None	20	15/20 (75%)	0.625%
	30	60	Neomycin	20	15/20(75%)	0.42%
			None	20	14/20(70%)	0.5%

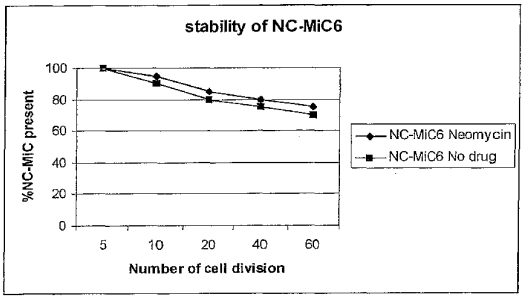

Figure 8

SEQUENCE LISTING

<110> AMRAD Operations Pty Ltd

<120> Neocentromere-based mini-chromosomes or artificial chromosomes

<130> 2478105/EJH

<140> not yet available
<141> 2001-12-20

<150> AU PR2247
<151> 2000-12-21

<150> AU PR8909
<151> 2001-11-16

<160> 2

<170> PatentIn version 3.0

<210> 1
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<212> DNA
<213> primer

<400> 1
ggattacagg yrtgagcca 19

<210> 2
<211> 18
<212> DNA
<213> primer

<400> 2
rccaytgcac tccagcct 18

【手続補正書】
【提出日】平成15年3月24日(2003.3.24)
【手続補正１】
【補正対象書類名】特許請求の範囲
【補正対象項目名】全文
【補正方法】変更
【補正の内容】
【特許請求の範囲】
【請求項１】
真核生物染色体に由来し且つネオセントロメアを含む単離された核酸分子であって、適合する細胞中でトランケーション型として存在し、又は適合細胞中へ予め作成されたＤＮＡ実体としてトランスフェクションにより導入された場合、複製でき、染色体外因子として作用でき且つ細胞分裂と共に分離できる核酸分子。
【請求項２】
真核生物染色体がヒト若しくは霊長類などの哺乳動物又は植物、鳥類、昆虫、ぜん虫、真菌、酵母又は爬虫類に由来するものである、請求項１記載の上記単離された核酸分子。
【請求項３】
真核生物染色体がヒトに由来するものである、請求項２記載の上記単離された核酸分子。
【請求項４】
真核生物染色体が家畜動物に由来するものである、請求項２記載の上記単離された核酸分子。
【請求項５】
該分子がマーデル（１０）染色体又はその等価物のｑ’腕の標的化されたテロメア関連トランケーション及びｐ’腕の無作為トランケーションの結合によって作成されるものである、請求項１記載の上記単離された核酸分子。
【請求項６】
該核酸分子が染色体１０又はその等価物の上のｑ２４とｑ２６の間に等しい位置にネオセントロメアを含むものである、請求項５記載の上記単離された核酸分子。
【請求項７】
該核酸分子が染色体１０又はその等価物の上のｑ２５に等しい位置に又はほぼｑ２５に等しい位置にネオセントロメアを含むものである、請求項６記載の上記単離された核酸分子。
【請求項８】
該核酸分子が染色体１０又はその等価物の上のｑ２５．２に等しい位置に又はほぼｑ２５．２に等しい位置にネオセントロメアを含むものである、請求項７記載の上記単離された核酸分子。
【請求項９】
ネオセントロメアに対応する領域がα－サテライトＤＮＡを実質的に欠いているものである、請求項１又は請求項６又は請求項７又は請求項８記載の上記核酸分子。
【請求項１０】
核酸分子が約０．５から約２．０Ｍｂのサイズである、請求項９記載の上記核酸分子。
【請求項１１】
核酸分子が約０．８から約１．６Ｍｂのサイズである、請求項１０記載の上記核酸分子。
【請求項１２】
ネオセントロメア又は機能性の相同染色体を同定する方法であって、哺乳動物のＣＥＮＰ－Ａ及び／又はＣＥＮＰ－Ｃに特異的な抗体又は哺乳動物のＣＥＮＰ－Ａ及び／又はＣＥＮＰ－Ｃと交差相互作用できる抗体を用いるクロマチン免疫沈降によりＤＮＡを単離する工程、免疫沈降により単離されたＤＮＡを増幅する工程且つ増幅されたＤＮＡの中に標識を組み込む工程、次いで増幅されたＤＮＡを用いてゲノムＤＮＡ又はその等価物を含むＤＮＡアレーを精査する工程、及び該免疫沈降したＤＮＡにハイブリダイズするクローンを

同定し単離する工程を含む方法。
【請求項１３】
哺乳動物がヒト、家畜動物、愛玩動物又は実験室試験動物である、請求項１２記載の上記方法。
【請求項１４】
哺乳動物がヒトである、請求項１３記載の上記方法。
【請求項１５】
ネオセントロメアが染色体１０又はその等価物の上のｐ２４とｐ２６の間に等しい位置にあるものである、請求項１４記載の上記方法。
【請求項１６】
ネオセントロメアが染色体１０又はその等価物の上のｑ２５又はほぼｑ２５の位置にあるものである、請求項１５記載の上記方法。
【請求項１７】
ネオセントロメアが染色体１０又はその等価物の上のｑ２５．２又はほぼｑ２５．２の位置にあるものである、請求項１６記載の上記方法。
【請求項１８】
ヒトのネオセントロメアに基づくミニ染色体（ＮＣ－ＭｉＣｓ）の形態の上記単離された核酸分子であって、該ＮＣ－ＭｉＣｓが細胞分裂の間に安定に分離できるネオセントロメア又はその潜在型、合成型若しくはハイブリッド型を含むものである核酸分子。
【請求項１９】
マーデル（１０）染色体又はその等価物のｑ’腕の標的化されたテロメア関連トランケーション及びｐ’腕の無作為トランケーションの結合によって作成される、請求項１８記載の上記単離された核酸分子。
【請求項２０】
ネオセントロメアが染色体１０又はその等価物の上のｑ２４とｑ２６の間に等しい位置にあるものである、請求項１９記載の上記単離された核酸分子。
【請求項２１】
ネオセントロメアが染色体１０又はその等価物の上のｑ２５又はほぼｑ２５の位置にあるものである、請求項２０記載の上記単離された核酸分子。
【請求項２２】
ネオセントロメアが染色体１０又はその等価物の上のｑ２５．２又はほぼｑ２５．２の位置にあるものである、請求項２０記載の上記単離された核酸分子。
【請求項２３】
ミニ染色体を作成する方法であって、
ネオセントロメアを含む染色体を保持するヒト若しくは哺乳動物の細胞の中に、テロメア配列、選択可能マーカー、標的染色体のｑ’腕又はｐ’腕の一方又は他方の上の相同なターゲティングＤＮＡを有するベクターを含むトランケーション構築物を導入する工程、
選択可能マーカーを発現する細胞を選択する工程、
該細胞に、該ｑ’又はｐ’ターゲティングＤＮＡの他方を含む第２のトランケーション構築物を導入する工程、
該第２のトランケーション構築物と関連する選択可能マーカーを発現する細胞を選択する工程、
次いで、ネオセントロメアを含むトランケーションされた染色体を単離する工程、
を含む方法。
【請求項２４】
細胞がヒトの細胞である、請求項２３記載の上記方法。
【請求項２５】
ネオセントロメアが染色体１０又はその等価物の上のｑ２４とｑ２６の間に等しい位置にあるものである、請求項２４記載の上記方法。
【請求項２６】

ネオセントロメアが染色体１０又はその等価物の上のｑ２５又はほぼｑ２５の位置にあるものである、請求項２４記載の上記方法。
【請求項２７】
ネオセントロメアが染色体１０又はその等価物の上のｑ２５．２又はほぼｑ２５．２の位置にあるものである、請求項２４記載の上記方法。
【請求項２８】
ネオセントロメアを挟んでいるｑ’及びｐ’腕を有する染色体を含む単離された細胞であって、ｑ’及び／又はｐ’腕の一方又は両方がトランケーションされているものである細胞。
【請求項２９】
受託番号００１２２００１（ＣＨＯ／ＢＥＺＢ３０）としてＥＣＡＡＣに寄託された単離された細胞系統。
【請求項３０】
受託番号００１２２００２（ＨＴ１０８０－ＭＩＣ１）としてＥＣＡＡＣに寄託された単離された細胞系統。
【請求項３１】
受託番号００１２２００３（ＨＴ１０８０－ＭＩＣ２）としてＥＣＡＡＣに寄託された単離された細胞系統。
【請求項３２】
受託番号００１２２００４（ＨＴ１０８０－ＭＩＣ３）としてＥＣＡＡＣに寄託された単離された細胞系統。
【請求項３３】
受託番号００１２２００５（ＨＴ１０８０－ＭＩＣ４）としてＥＣＡＡＣに寄託された単離された細胞系統。
【請求項３４】
受託番号００１２２００６（ＨＴ１０８０－ＭＩＣ５）としてＥＣＡＡＣに寄託された単離された細胞系統。
【請求項３５】
受託番号００１２２００７（ＨＴ１０８０－ＭＩＣ５ａ）としてＥＣＡＡＣに寄託された単離された細胞系統。
【請求項３６】
受託番号００１２２００８（ＨＴ１０８０－ＭＩＣ５ｂ）としてＥＣＡＡＣに寄託された単離された細胞系統。
【請求項３７】
遺伝子治療に使用するためのミニ染色体の製造における請求項１記載の単離された核酸分子の使用。
【請求項３８】
ミニ染色体がＮＣ－ＭｉＣである請求項３７記載の使用。
【請求項３９】
ミニ染色体がＹＡＣである請求項３７記載の使用。
【請求項４０】
ミニ染色体がＨＡＣである請求項３７記載の使用。
【請求項４１】
ミニ染色体がＭＡＣである請求項３７記載の使用。
【請求項４２】
ミニ染色体がＰＬＡＣである請求項３７記載の使用。

【国際調査報告】

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU01/01644

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: C12N 15/64, 15/79, 15/81, 15/82, 15/85

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
SEE ELECTRONIC DATABASES BELOW

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPIDS CA MEDLINE BIOSIS
Neocentromere, artificial chromososme, minichromosome, YAC, BAC, MAC, HAC.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98/51790 A1 (AMRRAD OPERATIONS PTY LTD) 19 November 1998	All
P X	Saffery, R; Wong, L;, Irvine, D; Bateman, M; Griffiths, B; Cutts, S; Cancilla, M, Cendron, A; Stafford, A; Choo, K. Construction of neocentromere-based human minichromosomes by telomere-associated chromosomal truncation. Proc Nat Acad Sci, USA. May 8, 2001. **98**(10): 5705-5710	All
Y	Mills,W; Crichter, R;b Lee, C; Farr, C. Generation of an~2.4Mb human X centromere-base minichromosome by targeted telomere-associated chromosome fragmentation in DT40 Human Mol Genet. 1999. **8**(5): 751-61.	All

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search
12 February 2002

Date of mailing of the international search report 18 FEB 2002

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU01/01644

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	Warburton P E. Making CENs of mammalian artificial chromosomes. Molecular Genetics and Metabolism. 1999. **68**(2): 152-60.	1-4, 12-14, 18-24, 37-42 5-11, 15-17, 25-36
Y	Cancilla M R; Tainton K M; Barry A E; Larionov V; Kouprina N; Resnick M A; Sart D D; Choo K H. Direct cloning of human 10q25 neocentromere DNA using transformation-associated recombination (TAR) in yeast. Genomics. 1998. **47**(3): 399-404.	5-11, 15-17, 25-36
A	Floridia G; Gimelli G; Zuffardi O; Earnshaw W C; Warburton P E; Tyler-Smith C. A neocentromere in the DAZ region of the human Y chromosome. Chromosoma. 2000. **109**(5): 318-27.	All
A	Williams B C; Murphy T D; Goldberg M L; Karpen G H. Neocentromere activity of structurally acentric mini-chromosomes in Drosophila. Nature Genetics. 1998. **18**(1): 30-7.	All
A	Grimes, B; Cooke, H. Engineering mammalian chromosomes. Hum Mol Genet. 1998. **7**(10):1635-1640	All

INTERNATIONAL SEARCH REPORT' Information on patent family members	International application No. PCT/AU01/01644

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Member
WO 98/51790 A1	AU A 73258/ 98
	EP A1 996719
	US A 626521

END OF ANNEX

Form PCT/ISA/210 (citation family annex) (July 1998)

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(51)Int.Cl.[7]	FI	テーマコード（参考）
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(81)指定国 AP(GH,GM,KE,LS,MW,MZ,SD,SL,SZ,TZ,UG,ZM,ZW),EA(AM,AZ,BY,KG,KZ,MD,RU,TJ,TM),EP(AT,BE,CH,CY,DE,DK,ES,FI,FR,GB,GR,IE,IT,LU,MC,NL,PT,SE,TR),OA(BF,BJ,CF,CG,CI,CM,GA,GN,GQ,GW,ML,MR,NE,SN,TD,TG),AE,AG,AL,AM,AT,AU,AZ,BA,BB,BG,BR,BY,BZ,CA,CH,CN,CO,CR,CU,CZ,DE,DK,DM,DZ,EC,EE,ES,FI,GB,GD,GE,GH,GM,HR,HU,ID,IL,IN,IS,JP,KE,KG,KP,KR,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD,MG,MK,MN,MW,MX,MZ,NO,NZ,OM,PH,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TN,TR,TT,TZ,UA,UG,US,UZ,VN,YU,ZA,ZM,ZW

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オーストラリア国，ビクトリア ３１４６，グレン アイリス，マクドナルド ストリート ７

Ｆターム(参考) 4B024 AA01 CA01 DA02 DA03 EA02 EA04 FA01 GA14
4B063 QA01 QA18 QQ42 QQ52 QR08 QR42 QR62 QR82 QS25 QS34
QS36 QX02
4B065 AA90X AA93Y AB01 BA01 BA24 CA44
4C084 AA13
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FI分类号	C12N15/00.ZNA.A A61K31/7088 A61K48/00 C12Q1/68.A G01N33/53.M G01N33/566 C12N5/00.A		
F-TERM分类号	4B024/AA01 4B024/CA01 4B024/DA02 4B024/DA03 4B024/EA02 4B024/EA04 4B024/FA01 4B024/GA14 4B063/QA01 4B063/QA18 4B063/QQ42 4B063/QQ52 4B063/QR08 4B063/QR42 4B063/QR62 4B063/QR82 4B063/QS25 4B063/QS34 4B063/QS36 4B063/QX02 4B065/AA90X 4B065/AA93Y 4B065/AB01 4B065/BA01 4B065/BA24 4B065/CA44 4C084/AA13 4C086/AA01 4C086/AA03 4C086/EA16 4C086/MA01 4C086/NA14 4C086/ZC80		
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优先权	2000PR2247 2000-12-21 AU 2001PR8909 2001-11-16 AU		
其他公开文献	JP4150588B2		
外部链接	Espacenet		

摘要(译)

本发明一般neocentromere或其功能衍生物或潜在形式涉及定义的或分离的核酸分子，其包含合成的或混合，并且特别是哺乳动物（例如人）和非一系列真核生物，包括哺乳动物微染色体和人工染色体微染色体及其在人工染色体发育中的应用。本发明提供了用于开发微染色体的端粒相关染色体截短（TACT）方法，但不限于这些方法。本发明涉及任何截断法来创建包含neocentromere微型染色体，并且还涉及使用克隆的或预先制备的DNA提供了在人工染色体的结构neocentromere功能的任何转染方法。这种微染色体和人工染色体可用于一系列基因治疗。