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ESTABLISHMENT OF A CONTINUOUSLY GROWING CHICKEN EMBRYO FIBROBLAST CELL LINE USING chTERT

Diplomarbeit zur Erlangung des akademischen Grades
Diplomingenieur der Lebensmittel- und Biotechnologie an der
Universität für Bodenkultur Wien

Eingereicht von
Herwig Stepan

Wien, September 2007

Danksagung

Diese Arbeit fasst nicht nur die Ergebnisse wissenschaftlicher Arbeit zusammen, sondern ist auch ein Symbol dafür, dass sich ein Lebensabschnitt dem Ende zuneigt. Nicht alleine sechsmonatiges Herumwuseln im Labor, sondern auch ein fünfjähriges Studium finden mit selbiger ihren Abschluss. Spätestens jetzt ist es Zeit all jenen zu danken, die ihren Teil zum Entstehen dieser Arbeit beigetragen haben, und die wohl während ihrer Entstehung nicht genügend mit Worten gewürdigt wurden.

Meine Zeit am IAM war eine äußerst lehrreiche, die mich sowohl fachlich als auch menschlich reifen ließ. Herrn Prof. Katinger ist einerseits dafür zu danken, dass er mit seinem Institut einen Raum geschaffen hat in dem wissenschaftlich produktive Forschung mit angenehmer zwischenmenschlicher Atmosphäre einhergehen, und andererseits dafür, dass auch ich für die Zeit meiner Diplomarbeit Teil der IAM-Familie werden durfte.

Weiters muss ich meinen beiden Betreuern Regina und Giovanni herzlichen Dank zukommen lassen. Giovannis fachliche Kompetenz mit der er stets mit Rat und Tat zur Seite stand und die gleichzeitigen Erzählungen von eigenen Rück- und Fehlschlägen während der eigenen Laborzeit, halfen sowohl wissenschaftlich als auch menschlich enorm weiter, um nach Misserfolgen die Pipette wieder in die Hand zu nehmen, und den nächsten verbesserten Anlauf zu starten. Auch Reginas Türe stand immer offen, und sie war stets eine Anlaufstelle, um sich Rat zur erfolgreichen Betreuung meiner Hühnerzellen zu holen, obwohl sie mit der Vorbereitung und Betreuung ihres ganz speziellen, eigenen Aging-Projektes sicher viel zu tun hatte.

Das „Laufen" im Labor brachten mir wohl „meine" beiden Post-Docs, Klaus und Matthias, bei. Meine ersten Schritte im vierten Stock wurden stets von Klaus begleitet, der viel Zeit in meine Betreuung investierte, und auch als ich das meiste alleine bewerkstelligen konnte immer da war, um die nächsten Versuche mit mir zu planen bzw. ihr Ergebnis mit mir zu diskutieren. Im dritten Stock, der Zellkultur, war es Matthias, der oft dazu verdonnert wurde meine Zellen anzuschauen und mir ihren ordnungsgemäßen Zustand zu versichern, oder andere Phänomene zu erklären.

Doch wie der Mensch nicht nur von Brot alleine lebt, so lebt der Wissenschafter auch nicht nur von Forschung. Ohne meine Mitdiplomanden, die meine Pausen und Inkubationszeiten bereichert haben, wäre es wohl nur eine halb so schöne Zeit gewesen. Mit Astrid, Alex, Martina und Michi hatte ich nicht nur immer eine lustige Zeit, sondern die Gespräche über ihre Arbeiten ließen mich auch über meinen CEF-Tellerrand hinausblicken.

Allen weiteren Mitarbeiter des IAM und der BOKU, die mir bei meiner Arbeit geholfen haben, sei an dieser Stelle kollektiv gedankt, um mich vor der nachträglichen Peinlichkeit einen vergessen zu haben zu bewahren. Viele Helferlein standen beim Einfrieren von Zellen, beim Suchen von Puffern, oder anderen Schwierigkeiten parat.

Zu guter Letzt sei all jenen zu danken, die vielleicht nicht unmittelbar mit der Arbeit zu tun hatten, aber mit meinem Studium oder Leben im Allgemeinen. Meinen Freunden, die mir stets zur Seite gestanden sind, und meiner Freundin, die mich durch das ganze Studium begleitete. Den besonderen Platz am Ende dieser Danksagung habe ich meinen Eltern freigehalten. Meiner Mutter, die mich stets mit allen ihr möglichen Mitteln unterstützt und gefördert hat, und meine Studienzeit durch viele „kleine“ Arbeiten, die meistens im Alltag untergehen, wesentlich vereinfacht hat. Und zuletzt meinem Vater, der diese Arbeit zwar nicht mehr lesen kann, aber doch wesentlich dazu beigetragen hat in dem er von frühesten Kindesbeinen meine Neugier und mein wissenschaftliches Interesse geweckt hat. Ich bin sicher er hätte eine „unheimliche Freud'“ damit gehabt!

Abstract

Primary cells have a limited life span, undergo senescence and stop proliferating after a certain number of cell divisions. This certainly poses a problem when they are used in a biotechnological production process. Having to change from primary culture to primary culture repeatedly is not only time and cost consuming but it also poses a threat on the stability of the process since mutations might be supported. Nowadays, many vaccines come from chicken egg-based production systems but due to long lead times and other disadvantages, they are very unflexible. This poses a great thret in case of a pandemic. Since Modified Vaccinia Virus Ankara (MVA), which was used as a vaccine against smallpox and nowadays has the chance to become an important recombinant live vaccine against other pathogens and for cancer therapy, was created by more than 500 passages on chicken embryo fibroblast (CEF) cells and therefore is highly adapted to this avian host, the idea was born to establish a continuously growing CEF cell line to ease MVA production.

Replicative senescence is caused by telemore shortening, which occurs in most cells except tumor, germ and embryonic stem cells. However, telomerase is known to be able to stop this process and therefore to prevent replicative senescence. Telomerase is a ribonucleo-protein consisting of two components: telomerase RNA, which is reported to be constitutively expressed in cells, and telomerase reverse transcriptase (TERT). Since attempts to immortalize CEF cells using human TERT have failed, and the sequence of chicken TERT (chTERT) was recently published, we wanted to attempt an immortalization using chTERT.

We amplified the chTERT sequence from cDNA of special chicken cells which are known to express high levels of chTERT and tried to introduce this sequence into a mammalian expression vector. This vector should have further been used to transfect primary CEF cells leading to the ectopic expression of chTERT. We hoped that this would lead to a significantly increased replicative life span allowing more population doublings or to a complete immortalization.

Unfortunately certain sections of the chTERT sequence (~4100 bp) caused severe trouble at PCR amplification and vector cloning. We were able to form a vector containing the rear ~3600 bp successfully and had the first ~500 bp synthesized. However formation of a vector

containing the complete chTERT gene was impossible due to different vector mutations when trying to introduce the whole gene.
As an alternate immortalization method, we tried the introduction of viral oncogenes of the Simian Virus 40 (SV40), large T and small t antigens. We were successful in transfecting CEF cells with SV40, which were then expressing viral proteins in the nucleus. However they did not show a growth advantage over normal CEF cells and did not lead to increased lifespan or immortalization.

We have shown difficulties at CEF immortalization which will hopefully ease future attempts to immortalize CEF cells.

Keywords: CEF, chTERT, telomerase, SV40, immortalization

Zusammenfassung

Primärzellen haben eine beschränkte Lebensdauer. Nach einer gewissen Anzahl von Zellteilungen hören sie auf sich zu teilen und werden seneszent, was natürlich ein großes Problem in biotechnologischen Produktionsprozessen darstellt. Immer wieder muss von einer Kultur auf die nächste umgestellt werden, was erstens Zeit und Geld kostet, und zweitens einen Unsicherheitsfaktor darstellt, da Mutationen dadurch verstärkt auftreten können. Eine Vielzahl der heutige gängigen Impfstoffe wird daher in Hühnereiern hergestellt. Aufgrund langer Produktionszeiten ist dies jedoch sehr unflexibel, und weißt zudem eine Reihe weiterer Nachteile auf. Modified Vaccinia Virus Ankara (MVA) beispielsweise wurde einst zur Impfung gegen Pocken eingesetzt und ist heute ein vielversprechender Kandidat, ein vielseitiger rekombinanter Impfstoff gegen andere Pathogene oder ein Mittel zur Krebstherapie zu sein. MVA wurde durch über 500 Passagen in Hühnerembryo-Fibroblasten (CEF) entwickelt, und ist daher hoch an CEF Zellen adaptiert. Um die Produktion von MVA erheblich zu vereinfachen sollte eine kontinuierlich wachsende CEF Zelllinie geschaffen werden.

Replikative Seneszenz wird in beinahe allen Zellen (außer Tumor-, Keim- und embryonalen Stammzellen) durch die Verkürzung der Telomere hervorgerufen. Allerdings kann dieser Prozess durch ein Enzym namens Telomerase verhindert werden. Es handelt sich um ein aus zwei Teilen bestehendes Ribonukleinprotein. Einerseits Telomerase RNA, die allerdings in der Regel konstitutiv exprimiert wird, und andererseits die Telomerase Reverse Transcriptase (TERT). Nachdem Versuche, CEF Zellen mit humaner TERT zu immortalisieren, gescheitert sind, und die Sequenz der Hühner-TERT (chTERT) kürzlich publiziert wurde, lag es nahe eine Immortalisierung mit chTERT zu versuchen.

Wir amplifizierten die chTERT Sequenz aus cDNA spezieller Hühnerzellen, die gewohnterweise hohe Konzentration an chTERT haben, und versuchten selbige in einen Expressionsvektor zu klonieren. Dieser Vektor sollte dann in weiterer Folge dazu verwendet werden primäre CEF zu transfizieren, und zu einer ektopischen chTERT Expression zu führen. Dadurch erhofften wir uns eine signifikante Verlängerung der replikativen Lebensspanne beziehungsweise sogar eine vollkommene Immortalisierung.

Unglücklicherweise verursachten bestimmte Abschnitte der chTERT Sequenz, die rund 4100 Basenpaare lang ist, große Probleme sowohl bei der PCR als auch beim Klonieren. Erfolgreich konnten wir einen Vektor klonieren, der den gesamten hinteren Teil (~3600 bp) in sich trägt. Die ersten 500 bp der Sequenz mussten synthetisiert werden. Allerdings kam es beim Zusammenführen dieser beiden Teile wiederholt zu Mutationen und Rekombinationen, was es letztendlich unmöglich machte das gesamte chTERT Gen in den Vektor einzubauen.

Als alternative Immortalisierungsmethode, versuchten wir virale Onkogene des Simian Virus 40 (SV40), large T and small t Antigene, in die Zellen zu bringen. Nach erfolgreicher Transfektion konnte die Proteinexpression der viralen Proteine im Zellkern festgestellt werden. Allerdings zeigten die transfizierten Zellen keine höhere Wachstumsrate und konnten die normalen Zellen letztlich nicht überwachsen. Es konnte weder eine verlängerte Lebensspanne noch eine Immortalisierung erreicht werden.

Wir haben einige Schwierigkeiten bei der Immortalisierung von CEF aufgezeigt, die zukünftige Bemühungen erleichtern könnten.

Stichworte: CEF, chTERT, Telomerase, SV40, Immortalisierung

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1. Introduction

1.1. Biotechnological vaccine production

Traditionally, a lot of vaccines, for example against influenza, are produced in embryonated chicken eggs. Therefore the virus is injected eleven days after fertilization into the eggs where it infects the embryo. Several days of incubation gives the virus time to propagate. Then the eggs are opened and the virus harvested and purified. Between one and two eggs are used to produce one dose of vaccine.

This method is well-established and cost effective but there are big disadvantages. A main problem is that millions of eggs don't grow immediately on demand. Due to the long production timeline of approximately six months, extensive planning has to precede the actual vaccine production. Since viruses can change their antigenic shape, the vaccines need to be constantly adapted. In case of the influenza virus, the World Health Organization (WHO) gives annual recommendations for the strains to be included into the vaccine. Due to this constant reformulation, influenza vaccines expire after one season. Currently the annual, worldwide, egg-based influenza vaccine production allows a worldwide coverage of only 17% (Ulmer et al., 2006). Almost five years of production would be necessary to deliver sufficient quantities of vaccine to allow a worldwide immunization. Another risk for egg-based production is the occurrence of viruses pathogenic to chicken. The depletion of chicken needed for egg production would have a drastic impact on vaccine production. Finally, the change of the composition of haemagglutinin after virus passage in eggs has to be mentioned (Oxford et al., 1987; Oxford et al., 1991; Robertson et al., 1987). This change from wild-type virus possibly leads to a less efficient vaccine (Wood et al., 1989).

All this contributes to the fact that egg-based production is not flexible enough in case of a pandemic. So there is great need for another production system, which can rapidly be scaled up in times of emergency. Mammalian cell culture seems to be a promising system. While vaccines for polio or mumps are already produced in cell culture, the production of numerous other vaccines is still in experimental stage. Resembling a biotechnological fermentation, mammalian cells are infected and propagate the virus as they divide. After harvesting and purification of the virus, a vaccine is gained which could be superior to vaccines produced in eggs. Since there is no egg albumin involved, there is no risk of causing allergies and also

other egg impurities are prevented. Another big advantage is that wild-type viruses can be safely propagated and there is no need to produce strains, which are attenuated to non-pathogenicity for eggs, which decreases the lead time for production considerably. The virus propagated in cell-based production systems is representative of the natural virus and it is reported that vaccines derived from mammalian cell culture causes equal or better protection than from egg-based production (Kistner et al., 1999; Nerome et al., 1999; Wood et al., 1989).

Besides influenza vaccine production, another field of application of cell-based vaccine production could be the production of Modified Vaccinia Virus Ankara (MVA). Highly attenuated viruses are used as vaccines against infectious diseases and cancer because they elicit a high immunoresponse without having the negative effects of the original virus. MVA was developed towards the end of the smallpox eradication campaign as a safe vaccine by Anton Mayr by long-term serial growth on chicken embryo fibroblasts (CEF) (Mayr et al., 1975). An important result was its loss of substantial genetic information, which led to a severe restriction of replication in mammalian cells, after 500 passages on CEF (Meyer et al., 1991; Drexler et al., 1998; Carroll and Moss, 1997). Therefore it is non-pathogenic for man and animals. Its genome size was reduced by 15% compared to the wild type precursor (Altenburger et al., 1989) and the complete sequence was analyzed (Antoine et al, 1998). In the future, it might play an important role as recombinant live vaccine against other pathogens and for cancer therapy. Since it is highly adapted to an avian host, the virus production in immortalized CEF cell line should be safe and considerably easier as well as time- and cost-saving compared to production in primary CEF cells. MVA production in alternate cell lines could possibly lead to viral mutations during the adaptation process.

1.2. Chicken embryo fibroblasts

The main hindrance to using CEF cells is that a technologically relevant line is missing due to replicative senescence. Since fresh primary cultures have to be produced continuously, CEF do not fulfil the demand of reliable and umlimited source. To ensure safety of primary cell cultures, they should be established from the endogenous virus free (EV-0) strain of white leghorns. This guarantees that there is no hazard of contaminating agents such as mycoplasma, bacteria or endogenous viruses. But due to expensive housing of specific pathogen-free animals and difficult and time-consuming cell culture establishment, this is no

profitable alternative. Currently there are three spontaneously immortalized cell lines (SC-1, SC-2, DF-1), which emerged from primary CEF culture after going through an extensive period of crisis (Himly et al., 1998; Christman et al., 2005; Christman et al., 2006). Furthermore, there are CEF cells available, which were immortalized by the introduction of viral oncogenes of the Simian Virus 40 (Bouquet et al., 2001). However, their use for biotechnological production processes is limited due to their changed phenotype and the lack of proper documentation.

1.3. Replicative senescence

Normal cells lacking telomerase activity can be maintained in vitro only for a limited number of population doublings (PD) before entering a phase of irreversible growth arrest caused by reaching a critical telomere length. Although they stop proliferating, most cells stay vital afterwards. This replicative senescence was first described by Hayflick and Moorhead (Hayflick and Moorhead, 1961). Chicken embryo fibroblasts, for example, have been cultivated until reaching 25-35 PD, which is their Hayflick limit (Hay, 1970; Venkatesan and Price, 1998).

1.4. Telomeres

Telomeres are non-nucleosomal DNA-protein complexes, which act as a cap for linear chromosomes. They are highly conserved, non-coding, hexameric sequences complexed with single- and double-strand binding proteins (reviewed in de Lange, 2002). Telomeric DNA of mammals and all other vertebrates consists of simple TTAGGG repeats with a 3′ overhang (50-100 bp), which is stabilized by the formation of a loop structure, known as the t-loop (Griffith et al., 1999). The telomere turns back on itself and the single strand overhang, which provides a single-stranded DNA target for binding telomere-specific proteins, forms a loop by inserting into the double helix as seen in Figure 1.1. TRFs (telomere repeat-binding factors) are found to bind and thereby stabilize the telomere loop structure. With this trick the telomeres "hide" their 3′ overhangs, which would resemble a double strand break (DSB) and would elicit a cell-cycle checkpoint response and an unwanted "repair" of the "break".

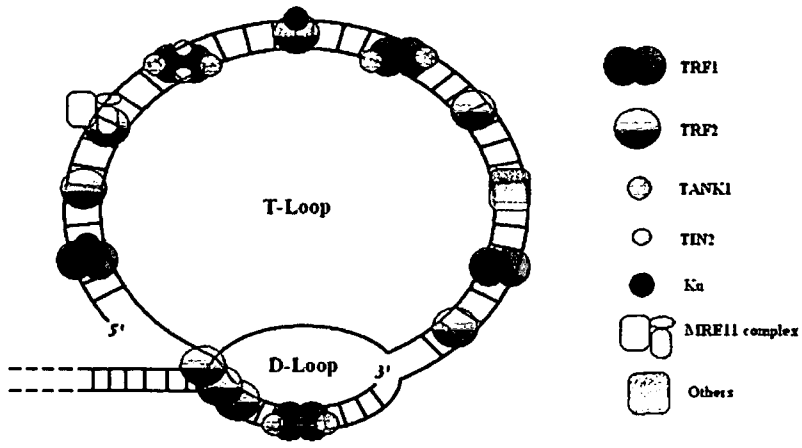

Figure 1.1 - T-loop structure with telomere specific proteins (Wai, 2004).

The primary function of telomeres is to maintain the integrity of the chromosome ends. Degradation, fusion of a telomere (either with another telomere or with a broken DNA end) and inappropriate recombination have to be avoided under all circumstances (Blackburn, 2005). This could lead to dicentric chromosomes, which induce imbalances in the genetic distribution of DNA at cell divisions, or simply to loss of genetic information. Therefore genome stability is crucially influenced by telomeres, and telomere erosion with successive cell divisions is a critical factor in controlling cell life span.

1.5. DNA replication and the end replication problem

To understand telomere erosion, DNA replication must be closely looked at.

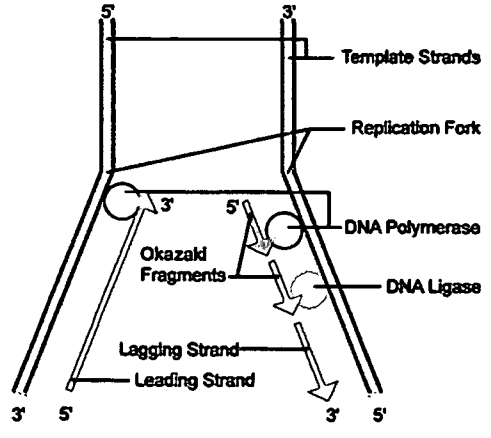

Figure 1.2 - DNA replication fork with leading strand and Okazaki fragments on lagging strand.

DNA replication in eukaryotes starts bi-directionally from an origin of replication, proceeds in 5′ and 3′ directions. The enzyme that copies the DNA sequences is DNA polymerase, which is unidirectional and polymerizes exclusively 5′→3′. Since the leading strand is replicated in this direction, DNA polymerase easily synthesizes a complementary strand. On the other hand, the lagging strand has to be replicated part by part. Short RNA templates work as primers when they attach to the lagging strand a little way ahead of the initiation site. The DNA polymerase starts replication from there and moves back to the initiation site. This procedure is repeated until the whole lagging strand has a complementary strand. Finally, DNA polymerase and DNA ligase work together to convert the RNA primers into DNA and to fill the gaps between the fragments (Figure 1.2). Unfortunately, the last RNA piece cannot be transformed into DNA and is degraded. Thus, at every replication the 5′ ends lose a short section. This phenomenon is called the "end replication problem" and was first described by Alexey M. Olovnikov in 1971 in a Russian journal (Olovnikov, 1971) and two years later in an American journal (Olovnikov, 1973), which thrust it into the full glare of worldwide publicity (reviewed in Levy et al., 1992; Klapper et al., 2001). Thus, the biochemistry of DNA replication results in failure to replicate 50-200 bp of 3′ telomeric DNA with each S phase (Campisi et al., 2001). Telomeres can be seen as a buffer, which prevent the loss of vital genetic information.

1.6. Telomerase and cellular immortalization

The enzyme that circumvents the end replication problem and maintains telomere length is a ribonucleo-protein called telomerase (Cong et al., 2002; Chan and Blackburn, 2004). It consists of two components: telomerase RNA (TR) and telomerase reverse transcriptase (TERT). TERT catalyzes the addition of nucleotides to the ends of the chromosome (Morin, 1989; Lingner et al., 1997). TR is the template for copying telomeric DNA repeats (Greider and Blackburn, 1989; Feng et al., 1995) and is reported to be constitutively expressed in human cells. That is why it is suggested that TERT limits the telomerase activity (O'Hare and Delany, 2005; reviewed in Poole et al., 2001).

Figure 1.3 shows how the TERT/TR-complex works together to elongate the 3′ overhang running 5′→3′ towards the distal end of the chromosome. Afterwards DNA polymerase can

generate the complementary strand without losing any repeats. A "telomere homeostasis" system prevents over-expression of telomeres or promotes telomeric extension depending on the actual telomere length (Levy and Blackburn, 2004). Since chicken has key features of telomere biology in common with man, it is an important higher vertebrate model in the study of telomerase activity (Forsyth et al., 2002; Delany et al., 2003).

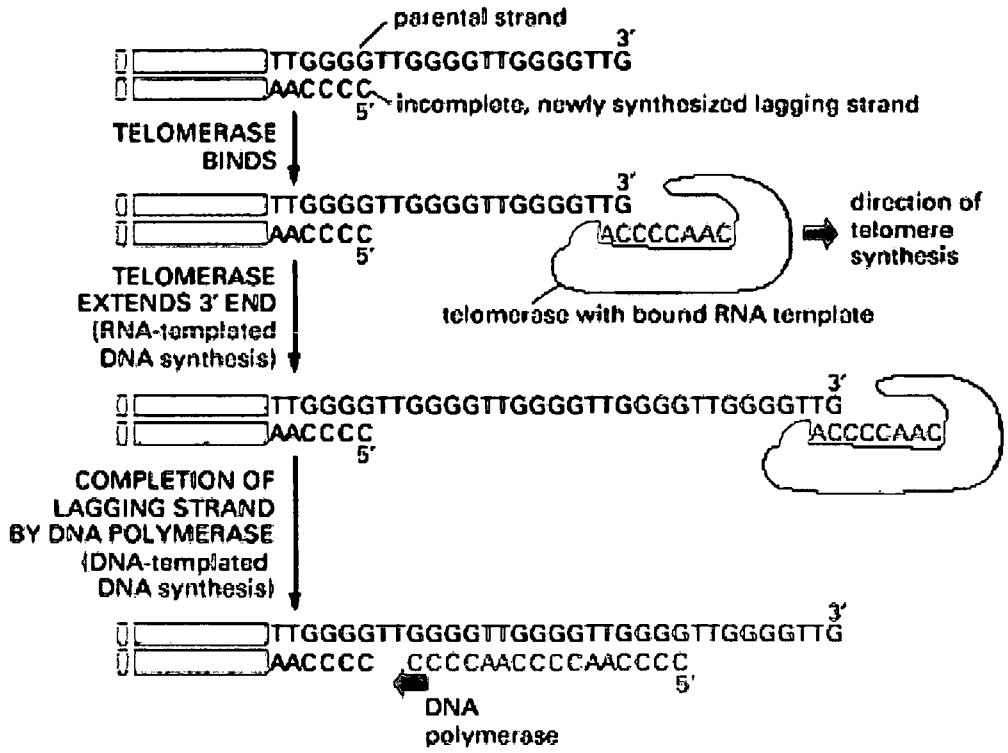

Figure 1.3 - Telomerase elongates the 3′ overhangs of telomeric ends in order to allow DNA polymerase to form a complete complementary strand without losing parts of the 5′ ends (Alberts et al., 2004).

Missing telomerase activity leads to shortening of telomeres with each replication. When a critical length is reached, telomeres become unable to form their t-loop structure and replicative senescence or apoptosis is evoked depending on the cell type (Beauséjour et al., 2003; Harrington and Robinson, 2002). This is why telomeres are called the cell's clock, which gives a hint on the replicative life span of a cell.

Most somatic human cells show low telomerase expression or none which explains why they are subject to cellular senescence. Their short telomeres are associated with replicative senescence, age-related diseases and premature aging syndromes (Blasco, 2005; Collins and Mitchell, 2002; Blasco et al., 1997). On the other hand, high levels of telomerase expression are found in most tumor cells (Kim et al., 1994; reviewed in Shay and Barchetti, 1997), and,

for example, germ cells and embryonic stem cells (Swanberg et al., 2004). Many surveys have shown that ectopic human TERT (hTERT) expression can immortalize eukaryotic cells by helping them to overcome senescence (Bodnar et al., 1998; Yang et al., 1999; Zhu et al., 1999; Chang et al., 2005) while not changing their phenotype (Carney et al., 2002; Jiang et al., 1999; Morales et al., 1999). This is a great advantage over other immortalization methods such as the introduction of viral oncogenes of Simian Virus 40 (Ray et al., 1990; Stewart and Bacchetti, 1991; Ohnuki et al.; 1996). Although chTERT is structurally similar to hTERT, the first 2 exons are significantly larger in chTERT and there are various levels of identity between the seven reverse transcriptase motifs in hTERT and chTERT (25% to 28%) (Fragnet et al., 2005). CEF cells have been reported to express very low levels of TERT (Venkatesan and Price, 1998) and therefore it is believed that TERT causes replicative senescence in CEF cells. Previous studies (Michailidis et al., 2005) have shown that the stable transfection of CEF cells with hTERT does not lead to increased telomerase activity nor to stabilization of telomere lenght. In 2004, the complete sequence of the chTERT gene was published (Delany and Daniels, 2004). This gives the opportunity to attempt to immortalize using chTERT.

2. Aims

The aim of this work was the establishment of a continuously growing chicken embryo fibroblast cell line using chTERT for vaccine production and other biotechnological applications.

This cell line should combine the phenotype of normal CEF cells with the advantages of a continuously growing cell line. Since it is reported (Chang et al., 2005; Voglauer et al., 2005; Jiang et al., 1999; Morales et al., 1999) that cell immortalization using human TERT maintains many characteristics of the corresponding parental cells which is a prerequisite for proper vaccine production, immortalization using the catalytic subunit of chicken telomerase was chosen.

Since the introduction of viral oncogenes of Simian Virus 40, large T and small t antigens has successfully led to the immortalization of human fibroma cells in our lab, we tried to this as an alternate immortalization method

3. Materials and Methods

3.1. Cell culture

3.1.1. Description of cell line - Chicken embryo fibroblast (CEF) cells

The cells used in this study were primary CEF cells isolated from specific pathogen-free (SPF) eggs from Baxter by Dietmar Katinger and frozen by Vera Gruber in our cell culture lab in March 2005.

The cells were cultivated in T flasks (NUNC, Denmark) with surface areas of 25 and 80 cm^2 in media volumes of 6 and 15 ml respectively. Ham's F12 basal medium (Biochrom AG, Berlin, Germany) supplemented with 4 mM L-glutamine and 10% fetal calf serum (FCS) (Pan-Biotech, Aidenbach, Germany) was used as medium. To allow better attachment of cells to the flasks, the flasks were coated with 1% gelatine in phosphate-buffered saline (PBS). Therefore, 1.5 ml of melted 1% gelatine in PBS were applied to a small T flask for 10-30 minutes and then poured away. Each flask was rinsed with PBS before inoculation. The common passaging routine started with removal of the old medium and washing the cells twice with PBS (without Ca^{2+}/ Mg^{2+}) to remove all FCS, which could inhibit trypsin. A solution of 0.1% trypsin and 0.02% ethylenediamine tetraacetic acid (EDTA) in PBS (prewarmed to 37°C) was added to the cells (e.g. 1 ml for a T25 flask) and poured off after the whole surface has been wetted. The T flask was then incubated at 37°C for 2-5 minutes to detach cells from the T flask.

Trypsin is a protease, which cleaves adhesion proteins, which allow the attachment of the cells to the T flask surface. The function of many of these proteins depends on cations like Ca^{2+} or Mg^{2+}. EDTA is a chelator, which binds bivalent cations and therefore leads to a concentration drop of free (solved) cations and cations that participate in protein/surface interactions.

After complete detachment of cells, the trypsin was inactivated by addition of fresh medium containing FCS. Finally, the cell suspension was transferred into new T flasks in the desired split ratio and new medium was added. Cells were cultivated at 37°C in an atmosphere of 5%

CO_2. To allow CO_2 equilibrium, screw caps were not screwed tightly for a minimum of 2 hours.

3.1.2. Calculation of cell number

Cell numbers of a roux flask were determined using a hemocytometer from Bürker-Türk was used. This device consists of a thick glass microscope slide with two railings in the middle, which hold a coverslip just 0.1 mm above an etched grid. This grid consists of nine large squares of exactly 1 mm^2 each (Figure 3.1). By placing the coverslip on the grid, chambers are formed, which hold 100 nl per large square. Capillary force is used to fill the chamber with sample after attaching the coverslip.

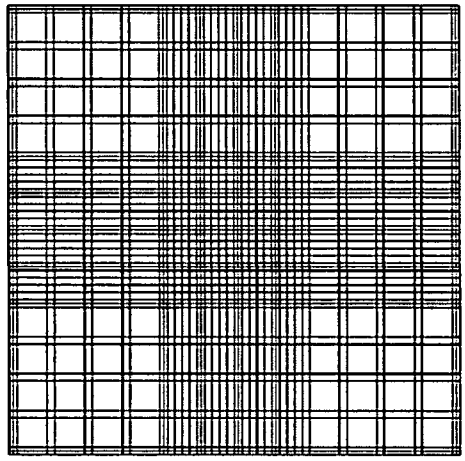

Figure 3.1 - Typical hemocytometer with 9 large squares.

Usually, 1 ml of cell suspension was mixed with 200 µl of trypan blue, which is a vital stain used to selectively color dead cells blue. After filling, the cells of five large squares were counted and used to calculate the cells/ml by multiplying the average cell count per large square by 12000. The number of viable cells and the viability of the cell suspension were determined using the following equations:

$$viable\ cells/ml = \frac{counted\ viable\ cells}{number\ of\ counted\ squares} x\ 12000$$

$$\%\,viability = \frac{viable\ cells}{total\ cell\ number} x100$$

3.1.3. Calculation of population doublings

One of the most important parameters of a cell line is its population doubling level (PDL), which reveals the age of a cell line by telling how often a single cell has divided. Roughly, population doublings (PD) can be estimated by using the split ratio. For example, when a confluent roux flask is passaged using a split ratio of 1:2, cells undergo one doubling until the cells are confluent again. However, for exact calculation the cell count of each roux flask must be determined before passaging to avoid mistakes by passaging at different confluency levels. By knowing how many cells have been plated (N_0) and knowing the cell count of a roux flask (N), exact calculations are possible. The PDL is a function of cumulative population doublings calculated by using this equation:

$$PD = \frac{\log\left(N/No\right)}{\log 2}$$

To establish a growth curve, the PDL was plotted against the days of cultivation.

3.1.4. Freezing cells

To save cells at certain PD levels, samples were frozen in liquid nitrogen. The cells of one confluent T80 flask were detached as described in 3.1.1 and resuspended in their normal culture medium. Centrifugation was performed at 170 g for 10 minutes to create a cell pellet and completely remove excessive medium. A special pre-cooled (4°C) cryo-conservation medium was added to ensure that freezing does not harm cells: 8 ml of Ham's F12 were mixed with 1 ml of FCS and 1 ml of dimethyl-sulfoxide (DMSO, Sigma-Aldrich Chemie GmbH, Steinheim, Germany), which prevents formation of harmful ice crystals during the freezing process. 3 ml of this special medium were used to resuspend the cells of one T80 flask, which were then filled into 3 ice-cooled cryo tubes. The cryo tubes were frozen at –80°C overnight and then transferred to the liquid nitrogen tanks.

3.1.5. Thawing cells

When thawing cells, one cryo tube was taken to inoculate one T25 flask. The vial was first transferred from the liquid nitrogen into a 50 ml-tube filled with 70% ethanol and then taken to the laminar flow hood. There the cells were thawed quickly by warming with hands, and then transferred into a prepared 10 ml tube filled with 7 ml of basal CEF medium to dilute the DMSO. The suspension was then centrifuged at 170 g for 10 minutes to pellet the cells and to completely remove the DMSO. The pellet was resuspended in 6 ml of basal medium and then transferred to one precoated T25 flask.

3.1.6. Determination of the toxic concentration of hygromycin

After transfection of cells with an antibiotic resistance gene including vector, successfully transfected cells are selected be applying the proper antibiotic to the culture. Since the vectors that we intended to use included the hygromycin resistance gene, we wanted to use geneticin sulphate (G 418) as selection agent. To test the toxic concentration of G 418 on non transfected cells, we added different concentrations of G 418 (20, 50, 100, 200, 400 µg/ml) to the culture medium. The lowest concentration that led to the death of all cells within 14 days, 100 µg/ml, was chosen.

3.1.7. Gene transfer of SV 40: lipofection

Lipofection is a method for gene delivery using non-viral vectors. A plasmid containing the gene of interest is attached to liposomes, which are spherical vesicles consisting of a phospholipid membrane. This membrane can merge with the cell membrane whereby the plasmid can be delivered to the cytoplasm.

The used plasmid, pDept, carries the early region of SV40 (largeT/smallt), including the SV40 promoter/enhancer sequences and the SV40 origin of replication which were inserted into the bacterial plasmid pBR322 by BamHI/KpnI restriction (Banerji et al., 1983).

Lipofection was performed using Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA). Cells were seeded into T25 flasks 24 hours prior to transfection to

reach about 70% confluence at transfection. For one T25 flask, 8 µg plasmid DNA were diluted in 500 µl of Opti-MEM Reduced-Serum Medium (GIBCO/Invitrogen, Paisley, UK). 20 µl of Lipofectamine 2000 Transfection Reagent were softly mixed with 500 µl Opti-MEM Reduced-Serum Medium and incubated for 5 minutes at room temperature. Then both solutions were mixed together and incubated for 20 minutes at room temperature to allow formation of DNA-Liposome complexes. After that, the medium was changed to 5 ml Opti-MEM Reduced-Serum Medium, and the prepared DNA-Lipofectamine solution was added. Cells were incubated for 6 hours at 37°C and 5% CO_2 before the medium was changed to normal growth medium.

Since there is no selective marker on the SV40-containing plasmid, we were unable to isolate successfully transfected cells, for example, by applying selection pressure of G418, and so we hoped that the growth enhancing properties of SV40largeT antigen would be sufficient to enable a selection of transfected cells by overgrowing untransfected cells.

3.1.8. Intracellular detection of proteins

To evaluate the efficiency of transfection with SV40largeT antigen, we marked SV40-expressing cells with a fluorescent stain and analyzed the percentage of stained cells by flow cytometry.

First, at least $5x10^5$ cells were detached from the flask and pelleted in a 10 ml-tube by centrifugation at 200 g for 10 minutes. Then 1 ml of 4°C cold 70% ethanol was added while vortexing. The tube was left for at least 1 hour at 4°C to ensure proper fixation of the cells. After that, ethanol was removed by centrifugation at 200 g for 10 minutes. The cells were washed with 1 ml of DNA buffer and 1 ml of DNA buffer containing 10% FCS; the centrifugation was performed as before. Then 100 µl of DNA buffer/10% FCS were added to saturate unspecific binding sites during twenty minutes of incubation at 37°C. 98 µl of DNA buffer/10% FCS were mixed with 1 µl of antibody 1 and 1 µl of antibody 2 (mouse anti-SV40largeT). Two antibodies against different epitopes of SV40largeT antigen were used in a 1:100 dilution to ensure proper detection after 1 hour of incubation at 37°C. Another washing step with 1 ml of DNA buffer/10% FCS was performed to remove the antibodies afterwards. 2 µl of secondary antibody (anti-mouse FITC conjugate) were mixed with 198 µl of DNA buffer/10% FCS and added to the cell pellet. Protected from light, the cells were left to

incubate for 1 hour at 37°C. At last, the cells were washed with 1 ml of DNA buffer to remove the secondary antibody and resuspended in 200 µl of PBS. The percentage of stained cells was analyzed by flow cytometry.

3.1.9. Immunofluorescence of adherent cells

The percentage of SV40-transfected cells was determined as described in 3.1.8. However, this method cannot be used to show where the protein is expressed within the cell. To prove the expression within the nucleus, we did a fluorescent staining of adherent cells which was then analyzed by confocal microscopy.

200 µl of cell suspension containing about $5x10^4$ cells were pipetted onto a sterile cover slip and incubated for 2-4 hours at 37°C to allow cells to attach to the cover slip. Additional medium was added to grow cells until subconfluence should have been reached. In the case of CEF cells, only few cells attached which was possibly caused by the lack of gelatine coating. Then the cells were washed twice with PBS and chilled (-20°C) methanol was used to fix them. After 5 minutes, the methanol was removed and the cells were dried at room temperature. To begin antibody incubation, the cells were soaked in PBS for 5 minutes before DNA buffer/10% FCS was added for 20 minutes to saturate unspecific binding sites. The cover slip was placed top down onto 50 µl of antibody solution (48 µl DNA buffer/10% FCS; 1 µl antibody 1; 1 µl antibody 2 of mouse anti-SV40largeT) and incubated at 37°C for 1 hour. Then the cover slip was washed three times for 5 minutes with DNA buffer/10% FCS. The incubation with the second antibody (anti-mouse FITC conjugate) and the washing steps were performed analog to the first antibody incubation. After the last washing step, the cover slip was carefully dried and placed with the cells facing downwards onto a drop of citifluor on a microscope slide. The analysis was performed using a Leica confocal microscope.

3.2. Molecular biology

3.2.1. General methods

3.2.1.1. *Polymerase chain reaction (PCR)*

Polymerase chain reaction is a quick and easy method for isolating and exponentially amplifying a specific fragment of DNA via enzymatic replication. The components of a typical reaction are:

- DNA template containing the region of interest
- Primers (synthetic oligonucleotides complementary to the 3′ ends of the target DNA segment)
- DNA polymerase (synthesizes DNA copy)
- Deoxynucleotide triphosphates (dNTPs)
- Buffer solution

The reaction is usually carried out in three steps (denaturation, annealing and elongation), which are repeated 20-35 times in a thermocycler. The main reaction is often preceded by one temperature hold in the beginning at 97°C for 1 minute to ensure that most of the DNA template and primers are denatured. After that cycling begins with one step at 94°C for 30 seconds (denaturation step). This step is followed by the annealing step where the temperature in the thermocycler is cooled down to 50-60°C, depending on the melting temperature of the primers. During this 30-second-step, stable bonds between the primers and the matching template sequence are formed. These short sections of double-stranded DNA are where the DNA polymerase attaches and begins DNA synthesis in the third step, which is called elongation step. Usually it is carried out at 72°C, which is close to the temperature optimum of DNA polymerase. Elongation time depends on the length of the sequence to be amplified, 1 minute per 1000 bases should be sufficient to get completely double-stranded DNA. In the end the main reaction is followed by one hold at 72°C for 3 minutes to fully extend any remaining single-stranded DNA.

During this work two different kinds of DNA polymerase were used: GoTaq DNA polymerase (Promega) and DNA polymerase (Biotools). Following reagents were mixed:

	Promega		Biotools	
Reagents	**Concentration**	**Added volume**	**Concentration**	**Added volume**
Sense primer	10 pmol/µl	0.5 µl	10 pmol	0.5 µl
Antisense primer	10 pmol/µl	0.5 µl	10 pmol	0.5 µl
Template	10-50 ng per 25 µl mixture	-	10-50 ng per 25 µl mixture	-
dNTPs	10 mM	0.5 µl	10 mM	0.5 µl
Buffer	5x GoTaq Green Master Mix	5 µl	10x Mg free buffer	2.5 µl
			$MgCl_2$ - 50 mM	1 µl
DNA polymerase	5 U/µl GoTaq DNA polymerase	0.25 µl	1 U/µl Biotools DNA polymerase	0.5 µl
AD		18.25 µl		19.5 µl
		25 µl		25 µl

Table 3.1 - PCR reaction mixture.

3.2.1.2. *cDNA synthesis*

First strand cDNA was prepared from a frozen total RNA isolation out of DT40-cells using SuperScript III reverse transcriptase (Invitrogen). This enzyme generates complementary DNA (cDNA) from single strands of mature mRNA. Following reagents were mixed:

Reagents	**Concentration**	**Added volume**
RNA isolation	1 µg/µl	5 µl
dNTPs	10 mM	1 µl
$Oligo(dT)_{18}$	50 µM	1 µl
AD		6 µl
	Incubate for 5 minutes at 70°C and for 1 minute on ice	
1st strand buffer	5x	4 µl
DTT	0.1 M	1 µl
RNAse OUT	40 U/µl	1 µl
Superscript III reverse transcriptase	200 U/µl	1 µl
	Incubate for 1 hour at 50°C	
		20 µl

Table 3.2 - cDNA synthesis reaction mixture.

After 1 hour incubation, the entire 20 µl were mixed with 2 µl of 2.5 M NaOH to degrade RNA and incubated for 10 minutes at 37°C. To neutralize, 10 µl of 2 M HEPES were added, and mixed thoroughly. Purification was then performed using Promega's Wizard SV Gel and PCR Clean-Up System by adding 32 µl of membrane binding solution and proceeding as described in 3.2.1.8.

3.2.1.3. *Restriction digestion*

In 1978 the Nobel Prize in medicine was awarded to Daniel Nathans, Werner Arber and Hamilton Smith for their discovery of restriction enzymes. These are an important part of the restriction modification system, which is used by bacteria to protect themselves from foreign DNA such as bacteriophages. The working mechanism is that "own" DNA is marked and therefore protected by adding methyl groups, which are not recognized by the restriction enzymes while non-host DNA is digested when the restriction endonucleases cleave double-stranded DNA at specific points. Every restriction enzyme recognizes its own specific sequence, which is usually 4-8 pairs long and often palindromic and there are also differences in the cleavage sites. Most enzymes produce "sticky" (also called cohesive) ends with 3′ or 5′-overhangs but some others leave blunt ends behind. This discovery led to the development of recombinant DNA technology, which brought a whole lot of new possibilities to scientists. The first big success was the artificial production of human insulin for diabetics using genetically modified E. coli strands. All enzymes used within this study were bought from New England Biolabs and applied in the supplied buffer unless indicated otherwise, which guarantees optimal activity. To save time double digestions were performed when different cleavage sites were needed and one suitable buffer for both enzymes was available. Following reagents were mixed:

Reagents	Concentration	Added volume
Vector (pCIneo)	1 µg/µl	3 µl
Supplied buffer (NEB 4)	10x	5 µl
Enzyme (Sma I)	20000 U/ml	2 µl
BSA	100x	0.5 µl
AD		39.5 µl
	Digest for 2 hours at room temperature	

Table 3.3 - Typical 50 µl restriction digestion mixture.

3.2.1.4. *Blunt ending of DNA fragments*

As already described, most restriction enzymes produce sticky ends that can be religated quite well. Although the yield after ligation is significantly lower with blunt ends there is the big advantage that these ends are always compatible. Since it is not always possible to find or to use the same restriction site on the insert and vector of interest, it is sometimes very helpful to use two different enzymes that leave incompatible overhangs and use another enzyme afterwards. This removes the overhangs and leaves compatible blunt ends behind. This enzyme is called Klenow fragment, which is a large protein fragment of DNA polymerase I. While DNA polymerase I has a 5′→3′ exonuclease activity, which makes it unsuitable for most subcloning applications, Klenow fragment only exhibits a 5′→3′ polymerase activity and a 3′→5′ exonuclease activity. That means that protruding 3′ overhangs are digested while recessed 3′ ends are filled. Following reagents were mixed:

Reagents	**Concentration**	**Added volume**
PCR product (DNA insert)		50 µl
Buffer (NEB 2)	10x	6 µl
Klenow fragment	5000 U/ml	1 µl
dNTPs	10 mM	0.2 µl
AD		2.8 µl
	Incubate 15 minutes at room temperature	
	Inactivate 5 minutes at 70°C	
		60 µl

Table 3.4 – Blunt ending mixture.

3.2.1.5. *Calf intestinal alkaline phosphatase (CIAP)*

To prevent vector religation, and thus reduce vector background in cloning strategies, CIAP was used. This enzyme catalyses the removal of 5′ phosphate groups from DNA which makes it impossible for DNA ligase to form covalent phosphodiester bonds between the 3′ hydroxyl end and the 5′ phosphate end.

After heat inactivation of restriction enzymes, 1 µl of CIAP (NEB) was added to a 50 µl preparation and incubated for 1 hour at 37°C. Since CIAP is active in most restriction buffers a buffer change was not necessary.

3.2.1.6. *Polynucleotide kinase (PNK)*

Sometimes it is necessary to add phosphoryl groups to the 5′-hydroxyl terminus to allow DNA ligation, for example after treatment with Klenow fragment. T4 PNK catalyses the transfer of a phosphate group from ATP to the 5′ end of a DNA strand.

Blunt ending mixture was heat inactivated and 1 µl of T4 PNK (NEB) were added along with 0.6 µl of ATP and incubated at 37°C for 30 minutes.

3.2.1.7. *Agarose gel electrophoresis of DNA*

Agarose gel electrophoresis is used to separate DNA molecules by size. Since DNA is highly negatively charged in solutions near neutral pH because of the phosphate groups, it migrates towards the anode when an electric field is applied. The migration speed then correlates with the length of the DNA fragment and smaller pieces move faster than larger ones. Conformation of the DNA also plays a role here since supercoiled DNA runs faster than linear DNA of the same size. Detection of the bands within the gel is performed by ethidiumbromide, which intercalates into nucleic acids and produces a fluorescent signal when excited by ultraviolet light.
This method can conveniently be used to separate DNA fragments after restriction digestion in order to prove correct size after ligation or PCR or simply to remove buffers or enzymes from DNA isolations. Following reagents were mixed:

Reagents	Concentration	Added volume / amount
Agarose (electrophoresis grade)		1.2 g
TAE buffer	50x	2.4 ml
AD		120 ml
	Melt in microwave oven	
	Cool down to ~60°C	
EtBr-solution	10 mg/ml	5 µl
	Cast in gel chamber	

Table 3.5 - 1% TAE agarose gel preparation.

After complete polymerization the gel was submerged in 1x TAE buffer containing 3 mg/l EtBr. The samples were mixed with a 6x loading dye (Fermentas) and loaded on a 1% TAE agarose gel for extraction or detection of fragments sized between 500 and 10000 base pairs or on a 2% TAE agarose gel for fragments smaller than 500 bp. Various markers (New England Biolabs, Fermentas) were used according to the expected fragment sizes.
For preparative gels 90-100 V and for quantitative gels 115-125 V were applied for 45-90 minutes until the desired bands were well separated. Detection was performed with a UV Transilluminator (Biorad). To protect DNA from UV induced damage, it was tried to keep UV exposure as minimal as possible (Cariello et al., 1998; Gründemann and Schömig, 1996).

3.2.1.8. *DNA purification after PCR and restriction digestion and from agarose gel*

Promega's Wizard SV Gel and PCR Clean-Up System, which is based on purification by binding silica matrices, was used for DNA purification after PCR, restriction digestion or from agarose gel. DNA has the ability to bind silica in the presence of high concentrations of chaotropic salts. After removing these salts with an alcohol-based membrane wash solution, DNA can be eluted in low-ionic strength solutions such as TE buffer or water.

After gel electrophoresis the band of interest was cut out of the gel and transferred to an Eppendorf tube where 10 µl of membrane binding solution per 10 mg of gel slice were added. After incubation at ~60°C until the gel had completely dissolved the whole mixture was transferred to the supplied minicolumns. To clean PCR reactions or restriction digestions an equal amount of membrane binding solution was added and directly transferred to the minicolumns. After 1 minute incubation to completely bind DNA to the membrane, the sample was centrifuged at 16000 g for 1 minute by an Eppendorf microcentrifuge and the flow-through was discarded. After two washing steps using 700 and 500 µl of membrane wash solution and the same centrifugation procedure as before, the membrane was dried to evaporate residual ethanol before the DNA was eluted into a new Eppendorf tube using 50 µl of nuclease-free water. DNA was either used immediately or stored at –20°C.

3.2.1.9. *Quantification of DNA*

3.2.1.9.1. *Agarose gel electrophoresis*

Agarose gel electrophoresis cannot only be used to estimate the size of fragments but also to make an approximate quantification. Therefore the intensity of the band of interest is compared with the intensity of the bands of special DNA ladders, for example MassRuler (Fermentas).

3.2.1.9.2. *Photometer*

The concentration of nucleic acids can also be determined by measuring the optical density (OD) at 260 nm (nucleic acids) and 280 nm (protein). The ratio of $OD_{260/280}$ should be between 1.80 and 2.00 to prove that the solution is pure and without contaminants. During this work a BioPhotometer by Eppendorf was used.

3.2.1.10. *Ethanol precipitation of DNA*

To purify DNA or simply to increase DNA concentration, ethanol precipitation was performed.

The DNA solution was mixed with 1/10 volume of 3M Na-acetate and 1 µl glycogen. After that a 2.5-fold volume of 96% ethanol was added and the whole mixture was cooled at –20°C for at least 1 hour. To recover the DNA precipitate the solution was centrifuged at 16000 g at 4°C for 30 minutes to pellet the DNA. The supernatant was discarded carefully and the pellet was washed once with 70% ethanol. To remove any residual ethanol, which could inhibit ensuing reactions, the pellet was air-dried before resuspension in 5 µl of sterile water was performed.

3.2.1.11. *DNA ligation*

After restriction digestion and creation of sticky or blunt ends, vector and insert have to be combined to create complete circular plasmids that can be used for transformation since linear

DNA would be degraded in bacteria. For this DNA ligase is used, which forms covalent phosphodiester bonds between corresponding 3′ hydroxyl and 5′ phosphate ends. ATP is required for this reaction. For ligation of inserts into vectors a molar ratio between 1:5 and 1:10 of vector to insert is used. 150-200 ng of insert are usually suitable. The exact molar ratio is dependent on the sequence length of the insert, bearing in mind that shorter pieces ligate better than longer ones.

During this work T4 DNA ligase from New England Biolabs was used, which has a temperature optimum of 16°C but can also be used at room temperature. Manufacturer's recommendation on ligation time is two hours at 16°C, or 10-15 minutes at room temperature to ligate sticky ends. Although the use of 1 µl of T4 DNA ligase containing 400 U is recommended, tests during this study have shown that use of less DNA ligase leads to better results. Following reagents were mixed:

Reagents	Concentration	Added volume / amount
Vector		20 ng
Insert		200 ng
T4 DNA ligase	400 U/µl	0.2-1 µl
Buffer containing ATP	10x	2 µl
AD		ad 20 µl
	Incubate 10-15 minutes at room temperature	

Table 3.6 - Ligation mixture of vector and insert.

3.2.1.12. *Transformation of bacteria (E. coli)*

To amplify plasmids, either original vectors or vectors containing an insert after ligation, bacteria are used, which replicate the desired plasmids. To be stably maintained in a cell, every vector must contain an origin of replication (ORI), which allows a chromosome-independent replication. Since only a small number of cells are transformed with the desired vector, most vectors contain an antibiotic resistance gene, which gives transformed cells the ability to grow on media containing this antibiotic whereas untransformed cells are unable to grow on it. Ampicillin and kanamycin were used during this study for selection, depending on which vector was used for transformation.

To introduce foreign DNA into cells, laboratory procedures are used to make cell membranes permeable to DNA. Two different approaches are commonly used:

3.2.1.12.1. *Chemically competent cells*

After chilling cells in the presence of divalent cations such as Ca^{2+}, they become DNA-permeable during a short heat shock. Chemically competent cells were bought from Stratagene to ensure high transformation efficiency ($1x10^8$ transformants/µg DNA) after difficult ligation procedures. SoloPack® Gold Competent Cells are delivered in a single-reaction tube, which makes them very easy to use.

After thawing one tube of competent cells frozen at –80°C, 1 µl of β-mercaptoethanol was added, gently mixed and incubated for 10 minutes on ice. Then the DNA, dissolved in 5 µl of water, was added and the tube gently swirled. Thirty minutes incubation on ice is followed by the heat shock procedure, which is critical because changes in time or temperature alter efficiency dramatically. The tube was submerged for 60 seconds into a water bath of 54°C and immediately cooled on ice for another two minutes. 250 µl of preheat SOC broth were added before the tube was left for 1 hour at 37°C with shaking at 300 rpm. After that the whole or parts of the bacteria suspension were plated on LB agar plates containing antibiotics (100 µg/ml) and incubated top down overnight at 37°C or 28°C.

3.2.1.12.2. *Electrocompetent cells*

Another way to bring foreign DNA through cell membranes is to shock bacteria briefly using high-voltage electric pulses. Bacterial plasma membranes consist mainly of amphiphilic lipids that self-assemble into highly insulating structures. However, the lipid matrix can be disrupted by a strong external electric field, which causes aqueous pores in the membrane by which DNA is taken up and which are rapidly closed by natural mechanisms afterwards (Tsong, 1991).

Electrocompetent cells were prepared directly in our lab. A special E. coli strain (DH10β) was grown in 10 ml of LB media overnight at 37°C and 200 rpm. 8 ml of overnight culture were used to inoculate 800 ml of LB medium, which were incubated until the culture reached an optical density of 0.6-0.8 at a wavelength of 600 nm. After harvesting by centrifugation (4500 rpm, 15 minutes, 4°C), the bacteria were extensively washed with 800 ml of ice-cold 1 mM

HEPES buffer and centrifuged as before. Two washing steps using 400 and 200 µl of HEPES buffer followed. The pellet was resuspended in 200 ml buffer containing 1 mM HEPES and 10% glycerol and centrifuged again. Finally, the bacteria were resuspended in 3 ml 10% glycerol and 100 µl aliquots were prepared. The tubes were shock frozen with liquid nitrogen and stored at –80°C.

For transformation, one aliquot was thawed, mixed with ~1 ng of vector and filled into a chilled electroporation cuvette lined with a strip of aluminum foil on each side. A short electric pulse of 2.5 kV, 1000 Ω and 25 µF was applied before 900 µl of SOC broth were added. After 30 minutes at 37°C, 100 µl and 900 µl of the bacterial suspension were dispersed on LB agar plates containing the appropriate antibiotic and incubated top down overnight at 37°C or 28°C.

3.2.1.13. *PCR screening*

After overnight incubation, the agar plates were checked for colonies to see if the ligation and transformation may have worked. PCR screening was performed to verify that colonies had taken up the desired whole construct and not only empty vector to acquire antibiotic resistance. Specific primers were used that bind to vector sequences flanking the multiple cloning site (MCS) or to sequences within the insert. To prove the correct orientation of the insert within the vector an insert primer was combined with a vector primer.

Colonies were chosen with a sterile pipette tip and dabbed into a standard PCR mix (Table 3.1). After performing PCR, 10 µl reaction mixture were mixed with 2 µl 6x loading dye (not necessary when GoTaq Green Master Mix was used) and loaded onto a 1% agarose gel. The length of the DNA fragments after electrophoresis was used to determine whether a colony can be assumed to contain the correct vector-insert ligation product or not. Positive clones were amplified to perform a plasmid preparation.

3.2.1.14. *Plasmid preparation*

After finding positive clones using the PCR screening method, the desired vector has to be amplified and purified so that further work can be done. This can be either another restriction digestion or, for example, transfection of mammalian cells.

The crucial part of plasmid purification is the separation of plasmid DNA not only from proteins and lipids but also from chromosomal DNA and cellular RNA of the host bacteria. The basic steps are disruption of the cell structure, separation of soluble DNA from cell debris and other insoluble material and purification of plasmid DNA from soluble proteins and other nucleic acids.

Most purification kits are based on the SDS-alkaline denaturation method, which exploits the difference in denaturation and renaturation characteristics of covalently closed circular plasmid DNA and chromosomal DNA fragments. Under alkaline conditions (pH 11), both plasmid and chromosomal DNA are efficiently denatured. Rapid neutralization with a high-salt buffer such as potassium acetate in the presence of SDS causes the chromosomal DNA to base-pair in an intrastrand manner, forming an insoluble aggregate that precipitates out of solution, while the covalently closed circular plasmid DNA remains in solution. The precipitated and aggregated proteins also assist in the entrapment of the high-molecular-weight chromosomal DNA. Filtration or centrifugation are used afterwards to remove the insoluble compounds. The further purification of plasmid DNA is done by silica. A silica-based membrane in a column format is used to bind the plasmid DNA contained in the cleared alkaline lysates. Purification is based on selective adsorption of DNA to the silica membrane in the presence of high concentrations of chaotropic salts. Washing steps ensure the removal of any contaminants and finally plasmid DNA is eluted with low-salt solutions such as TE buffer or water. All steps were performed according to the manufacturer's protocol.

3.2.1.14.1. Miniprep (Wizard® Plus SV Minipreps DNA Purification System, Promega)

1-10 ml antibiotic-containing medium were inoculated with a positive colony after PCR screening by a sterile pipette tip and incubated overnight at 28°C or 37°C. The cells were harvested by centrifugation for 5 minutes at 10000 g in a tabletop centrifuge and the

supernatant was poured off, making sure to remove any excess media. 250 µl of Cell Resuspension Solution were added in order to completely resuspend the pellet after vortexing. 250 µl of Cell Lysis Solution containing 1% SDS and 0.2 M NaOH were added and the tube was gently inverted to prevent shearing of chromosomal DNA. After partial clearing of the lysate (incubation time 1-5 minutes), 10 µl of Alkaline Protease Solution were added and the suspension inverted four times and incubated at room temperature for 5 minutes. 350 µl of Neutralization Solution were added and the tube was immediately inverted before the bacterial lysate was centrifuged at 16000 g for 10 minutes at room temperature. The cleared lysate was then transferred to a silica spin column taking care not to disturb any of the white precipitate. The supernatant was centrifuged at 16000 g for 1 minute and the flow-through discarded. 750 µl of Column Wash Solution were added to the spin column, which was again centrifuged at 16000 g for 1 minute. Another washing step with 250 µl of Column Wash Solution followed; the flow-through was discarded and an additional centrifugation step of 2 minutes made to remove any residual ethanol. The spin column was then carefully transferred to a new sterile Eppendorf tube. 50 µl of nuclease-free water were added and left to stand for 1 minute. The last centrifugation at 16000 g for 1 minute was performed to elute the plasmid DNA, which was then directly used or stored at –20°C.

3.2.1.14.2. Midiprep and [Maxiprep] (PureYield® Plasmid Midiprep [Maxiprep] System, Promega)

50 [250] ml antibiotic-containing medium were inoculated with a positive colony after PCR screening by a sterile pipette tip and incubated overnight at 28°C or 37°C. Cells were pelleted by centrifugation at 5000 g for 10 minutes and the supernatant discarded. Excess medium was removed by draining tubes on a paper towel; 3 [12] ml of Cell Resuspension Solution were added and extensive vortexing made sure of completely resuspending the pellet. 3 [12] ml of Cell Lysis Solution were carefully added and the tube was gently inverted 3-5 times before 5 [12] ml of Neutralization Solution were added to the lysed cells. After inverting 5-10 times, the tube was centrifuged at 15000 g for 15 minutes to pellet the bulk of cellular debris. While the centrifuge was running, a column stack was assembled by nesting a clearing column into the top of a binding column. This stack was then assembled onto a vacuum manifold. After centrifugation the cleared lysate was carefully poured into the clearing column, which removed the remaining debris. Vacuum was applied until all the liquid had passed through both columns. After slowly releasing vacuum, the clearing column was discarded while the

binding column containing the plasmid DNA stayed on the manifold. 5 [5] ml of Endotoxin wash were added and pulled through the membrane before 20 [20] ml of column wash were applied in the same way. To remove any residual ethanol, the membrane was dried by applying a vacuum for about 1 minute until the membrane appeared to be dry and there was no detectable ethanol odor. The Binding column was then put into a new 50 ml plastic tube and 600 [1000] µl of nuclease-free water were added on top of the membrane to elute plasmid DNA. A centrifuge with a swinging bucket was used to centrifuge at 2000 g for 5 minutes. The filtrate from the 50 ml tube was then transferred to a 1.5 ml Eppendorf tube and stored at –80°C.

3.2.1.15. *Sequencing*

Chain terminator sequencing (Sanger sequencing) is the most common method of DNA sequencing. A primer complementary to the beginning of the sequence to be determined is used as the starting point from which a DNA polymerase extends a complementary strand. This is different from a normal PCR; not only deoxynucleotide (dNTP) but also some di-deoxynucleotide (ddNTP) bases are added, which serve as chain-terminating nucleotides. Labelled terminators are used in dye terminator sequencing where each chain terminator is labelled with a different fluorescent dye. These di-deoxynucleotides lack the OH group at the 3′ carbon and therefore cannot bind to another nucleotide which causes an elongation stop. As a result, strands of different lengths appear, which can be separated and the last base can be determined by scanning the fluorescent ddNTPs.

All sequencing during this work was performed by Martin IBL.

3.2.1.16. *Glycerol stocks of bacteria*

Stocks of transformed bacteria strains were prepared for maintenance and long-term storage.

100 µl of overnight culture grown in LB medium containing an antibiotic were mixed with 50 µl of sterile 87% glycerol and frozen at –80°C.

3.2.2. Cloning

Vector maps of all original vectors and sequences of all primer sequences are given in 8 Appendix. Primers were either designed using the PrimerSelect software (DNASTAR, Inc.) or according to the protocol of the vector manufacturer in the case of vector primers like T7 promoter.

3.2.2.1. *chTERT-vector construction*

Vector construction of a vector including the coding sequence of the chTERT gene was started with reversely transcribed RNA out of DT40 cells as a template. These cells are known to have a very high chTERT expression level (Swanberg et al., 2004). Owing to a high GC-content within the first 500 bp of the published chTERT sequence (Delany et al., 2004; Chang and Delany, 2006), which made PCR impossible, my predecessors on this project decided to have this part, later called the 5´-part, synthesized by AdriaCell Srl (Trieste, Italy). To simplify PCR amplification of the remaining part, it was chosen to amplify two parts with the primer pairs: chTERT512sense/chTERT2719AS and chTERT2564sense/chTERT4082AS. The enzymes ApaLI and HaeII were chosen to digest the parts and enable correct ligation.

3.2.2.2. *Exchange of antibiotic resistance genes of pSilencer 2.1-U6 hygro*

The vectors pSilencer 2.1-U6 hygro – SNEV, pSilencer 2.1-U6 hygro – miR193a and pSilencer 2.1-U6 hygro – luc1 were already available in our lab. The challenge was to find a suitable vector with a kanamycin resistance gene, extract it and use it to exchange the hygromycin/ampicillin genes.

3.2.2.3. *Transfer of pre-hsa-miR inserts from pSilencer 2.1-U6 hygro into pcDNA3.1 hygro (-)*

Four different pre-hsa-miR-constructs (miR21, miR29a, miR31 and miR193a) cloned into pSilencer 2.1-U6 hygro were available in our lab. BamHI and HindIII were used to extract the different inserts and the new vector pcDNA3.1 hygro (-) where they were ligated into.

4. Results

4.1. Cell culture

4.1.1. General data

After thawing, CEF cells (3.1.1) were passaged as described in 3.1.1 twice a week at a split-ratio of 1:2 and 1:4. This growth rate of about three population doublings (PD) per week was according to the data that has previously been acquired in our lab. After approximately two months of continuously passaging and reaching PDL 25, cells reached replicative senescence (Figure 4.1).

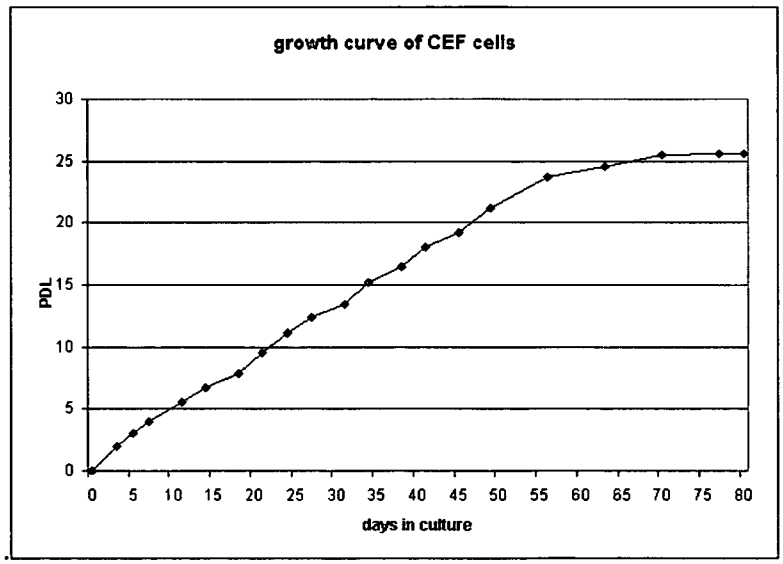

Figure 4.1 - Growth curve of CEF cells.

The proliferation rate slowed down significantly until cells completely stopped dividing. However, the senescent cells were fed with fresh medium once a week and stayed alive until this experiment was stopped, as postulated for senescent cells (Hayflick, 1961).

During the exponential growth phase, cells showed typical fibroblastoid morphology. The branched cytoplasm surrounding an elliptical nucleus was clearly visible during early stage passages. In contrast, senescent cells showed an enlarged and flattened morphology (Figure 4.2).

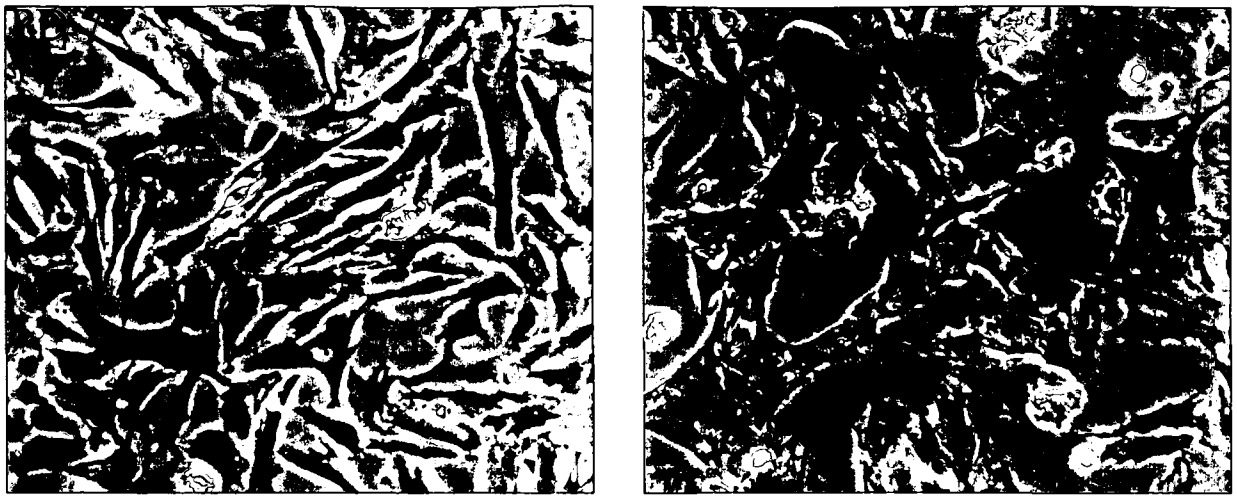

Figure 4.2 - CEF cells in exponential growth phase (PD7) and senescence (PD24).

4.1.2. Transfection of cells with early region of SV40 (largeT/smallt)

To try an alternate immortalization approach beside from the introduction of the chTERT gene coding sequence, we introduced viral oncogenes of the SV40, large T and small t antigens. As described in 3.1.7, we performed a lipofection with pDept on early passage CEF cells (PD3). Intracellular detection of expressed protein to evaluate the percentage of transfected cells was realized as described in 3.1.8 by staining the transfected cells with a fluorescent antibody and counting these cells by flow cytometry.

PD of control and SV40-tranfected CEF cells	SV40-positive cells
PD4/PD1pT	7%
PD6/PD3pT	1%
PD17/PD14pT	13%
PD21/PD18pT	0.5%

Table 4.1 – Percentage of SV40-expressing cells at various stages of exponential phase analyzed by flow cytometry.

As shown in Table 4.1, we found 7% of cells expressing SV40 and therefore positively transfected one PD after transfection. Since there is no antibiotic resistance gene on the used plasmid, the selection of transfected cells would happed due to a growth advantage of transfected cells which should overgrow non-transfected cells. At PD14pT we found the highest level of SV40 expressing cells. To prove the expression of the protein within the

nucleus, we performed a staining of adherent cells (3.1.9). Figure 4.4 shows pictures of the staining taken with a confocal microscope. Two cells clearly showed a strong fluorescence at the nucleus while others did not show any. This accords to the results we had from flow cytometry. Unfortunately our last analysis at PD18pT (Figure 4.3) did not show anymore significant levels of transfected cells.

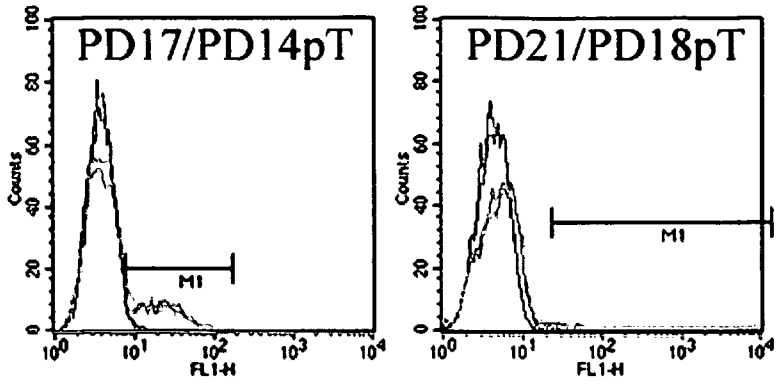

Figure 4.3 - FACS analysis of SV40-transfected and control cells which shows a strong decrease of transfected cells between PD14pT and PD18pT.

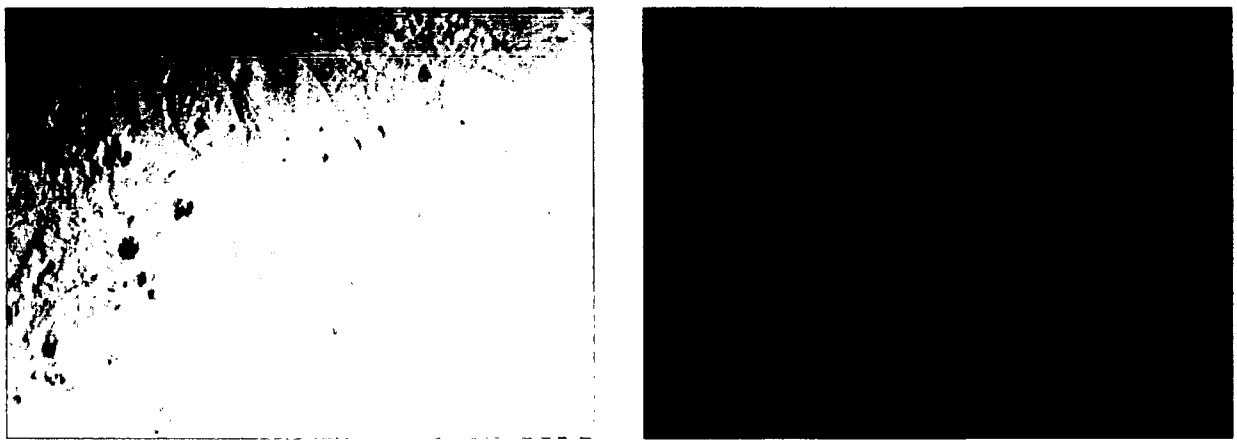

Figure 4.4 – Immunofluorescent staining (mouse anti-SV40largeT) - transfected cells (PD19/PD16pT) showing strong SV40 expression within the nucleus.

The morphology of transfected and control cells (Figure 4.5) and their growth rates (Figure 4.6) were continuously compared. Growth rates did not significantly differ and no growth advantage of the transfected cells was observed. This is a contrast to the results of immortalizing human fibroblasts using the same strategy (Voglauer et al., 2005). Due to the heterogeneity of cells within the control cells, it was quite difficult to determine transfected

from un-transfected cells. However, thin, elongated cells were found within the transfected cells much more often than within control cells.

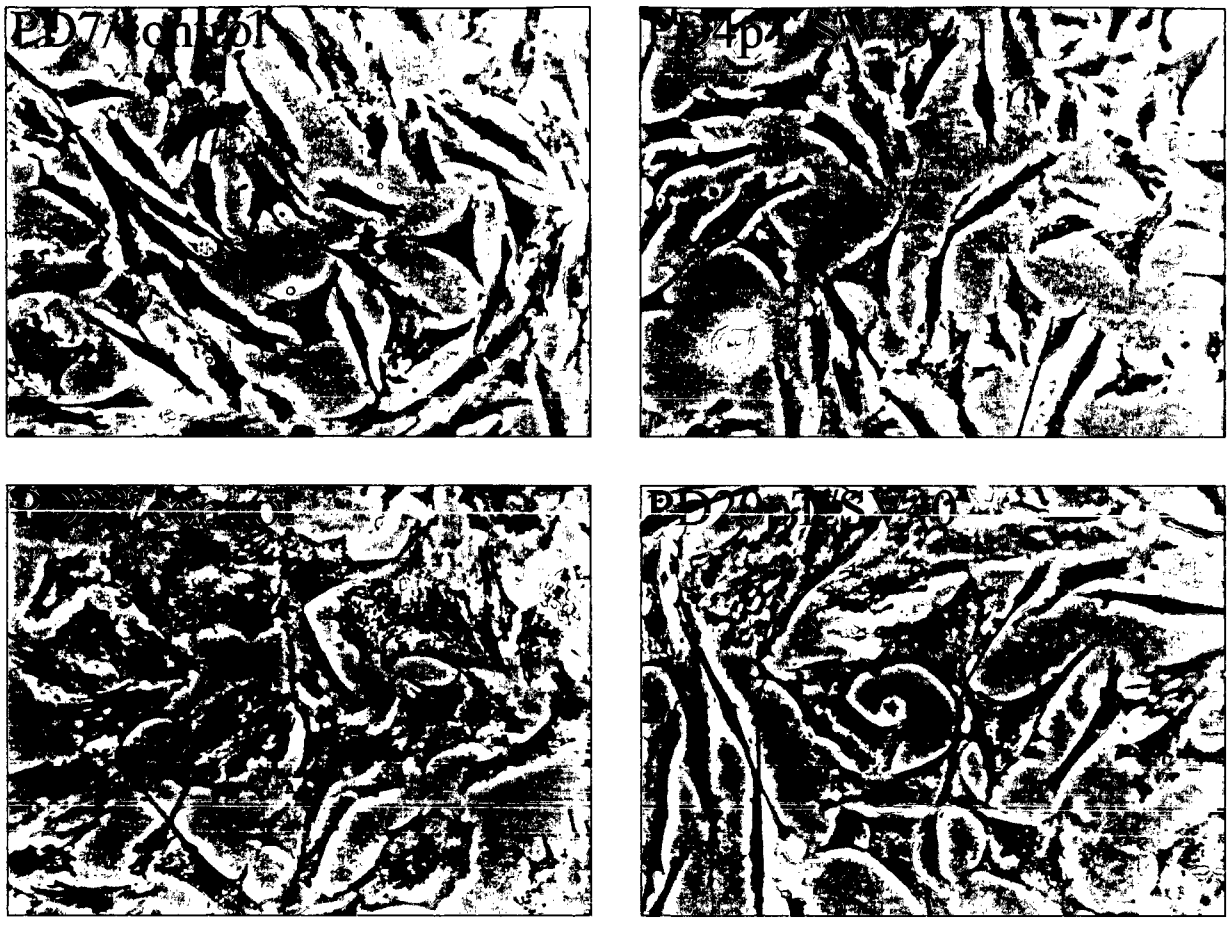

Figure 4.5 - Morphological changes of cells at different stages of exponential growth phase and differences between control and SV40-transfected cells.

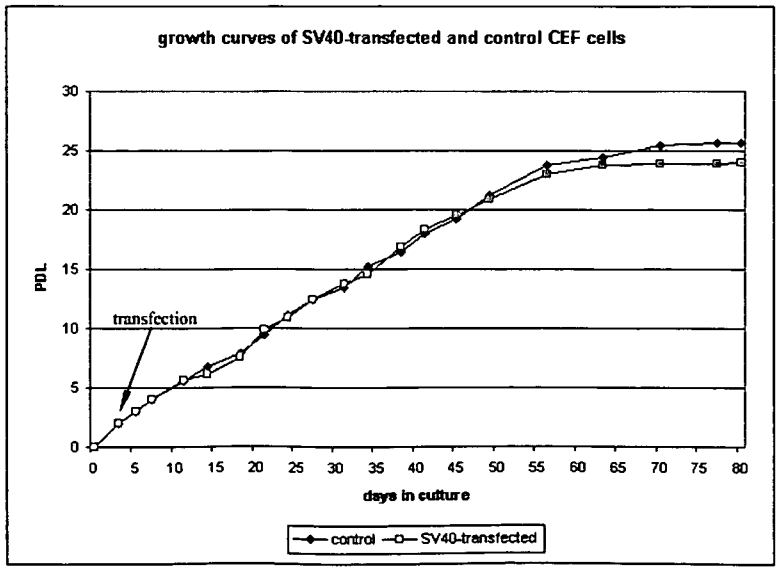

Figure 4.6 - Growth curves of SV40-transfected and control CEF cells.

4.2. Generation of genetic constructs: chTERT

The major aim of this study was the immortalization of CEF cells using the catalytic subunit of chicken telomerase, chicken telomerase reverse transcriptase (chTERT). Therefore, a total RNA isolation of DT40 cells was reverse transcribed. Then specific primers were used to amplify the chTERT gene. The last step was the ligation of the chTERT gene into a vector suitable for expression in mammalian cells.

4.2.1. Isolation of gene of interest

The chTERT gene was twice isolated from a DT40 cell line, which is known to express high levels of TERT (Swanberg and Delany, 2005). 5 µg of total RNA were taken for reverse transcription (3.2.1.2) in order to get enough cDNA for amplification.

The primary intention was to amplify the whole open reading frame (ORF), which is about 4000 bp long, using one single PCR of the known chTERT gene (NM_001031007, NCBI reference sequence).

Unfortunately, preliminary work of Dr. Klaus Fortschegger et al. has shown that this simple method was not practicable owing to regions of high GC-content at the beginning of the sequence, which inhibit proper PCR amplification. That is why we decided to divide the gene into three parts that were called 5′-, M- and 3′-part, to amplify the parts independently and ligate them together before ligating the whole gene into the chosen vector. The restriction sites of ApaII and HaeII, which are single cutters within the chTERT sequence, were selected to divide the gene.

The program PrimerSelect was used to design specific primers to amplify the three different parts.

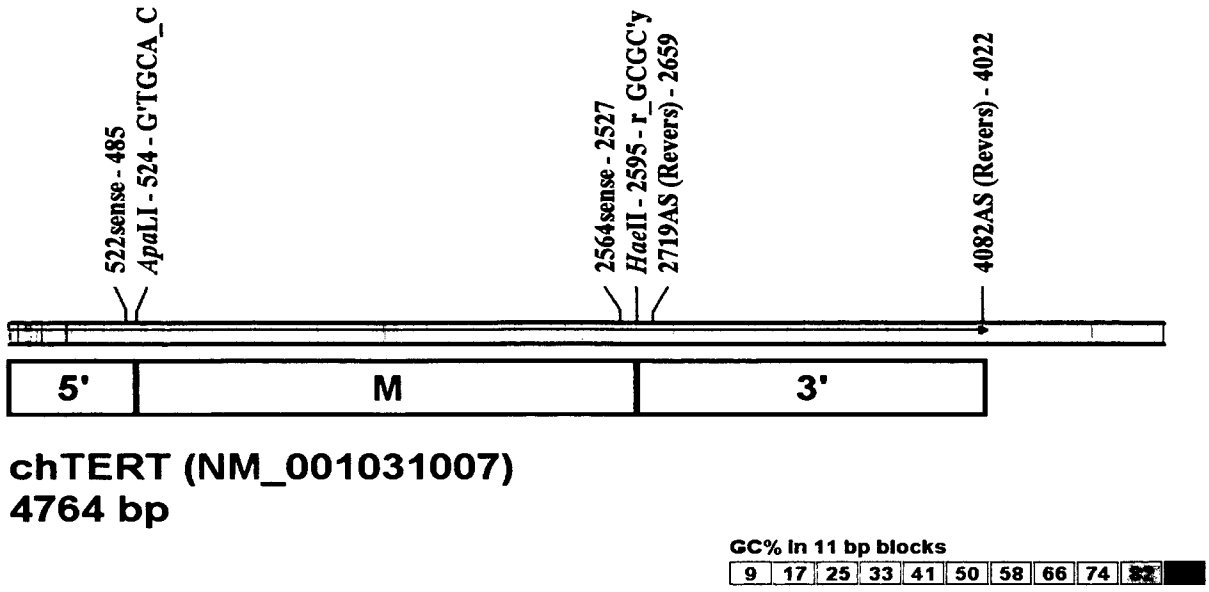

Figure 4.7 - chTERT gene divided into three parts for amplification

Although numerous primers were tried to amplify the 5′ region (data not shown here), PCR of this piece was never successful. To bypass this problem the 5′ piece was synthesized by AdriaCell Srl (Trieste, Italy). As shown in Figure 4.7 primers chTERT522sense and chTERT2719AS were used for the middle piece, and chTERT2564sense and chTERT4082AS for the 3′ piece.

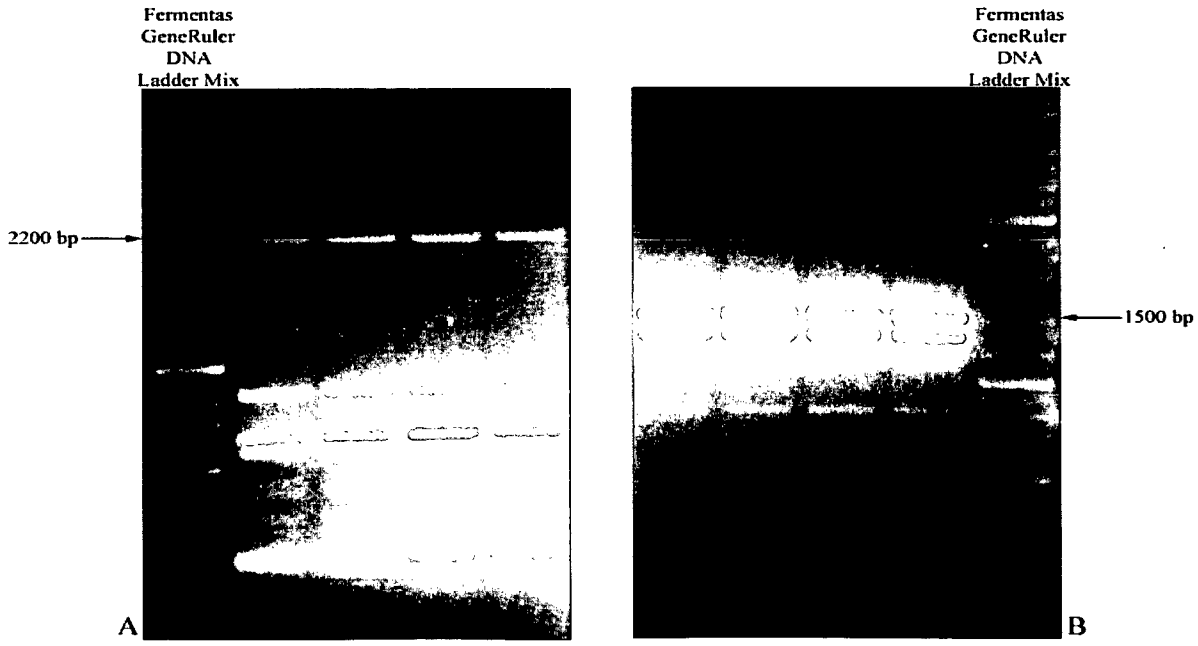

Figure 4.8 - PCR product on a 1% TAE-agarose gel. **A:** PCR of M-part - expected band at 2200 bp. **B:** PCR of 3′-part - expected band at 1500 bp.

Ten PCRs (50 µl mixture) were performed as described in 3.2.1.1 with 60°C annealing temperature and 2 minutes 30 seconds elongation time for the M-part, 5 PCRs (50 µl mixture) with 55°C annealing temperature and 2 minutes 30 seconds elongation time for the 3′-part.

Agarose gel electrophoresis was performed as described in 3.2.1.7 and DNA was extracted (3.2.1.8) before quantification (3.2.1.9.2). 0.8 µg of the M-part and 1.3 µg of the 3′-part could be purified. The rather small yield of the product of the M-part at 2200 bp might be caused by the numerous splicing variants, which were published (Chang and Delany, 2006).

4.2.2. Preparation of plasmid 1

To enable correct ligation of the gene into the chosen vector pCIneo, all three parts and the vector were digested with two different restriction enzymes. The 5′-part from the synthesized Adriacell-construct was digested with XhoI and ApaII (3.2.1.3), the M-part digested with ApaII and HaeII and the 3′-part was blunt ended (3.2.1.4) on one side and digested with HaeII on the other. The vector was digested with XhoI and SmaI, which creates blunt ends.

As shown in Figure 4.9 all fragments showed the expected size of ~530 bp (5′-piece), ~2070 bp (M-piece), ~1490 bp (3′-piece) and ~5440 bp (vector).

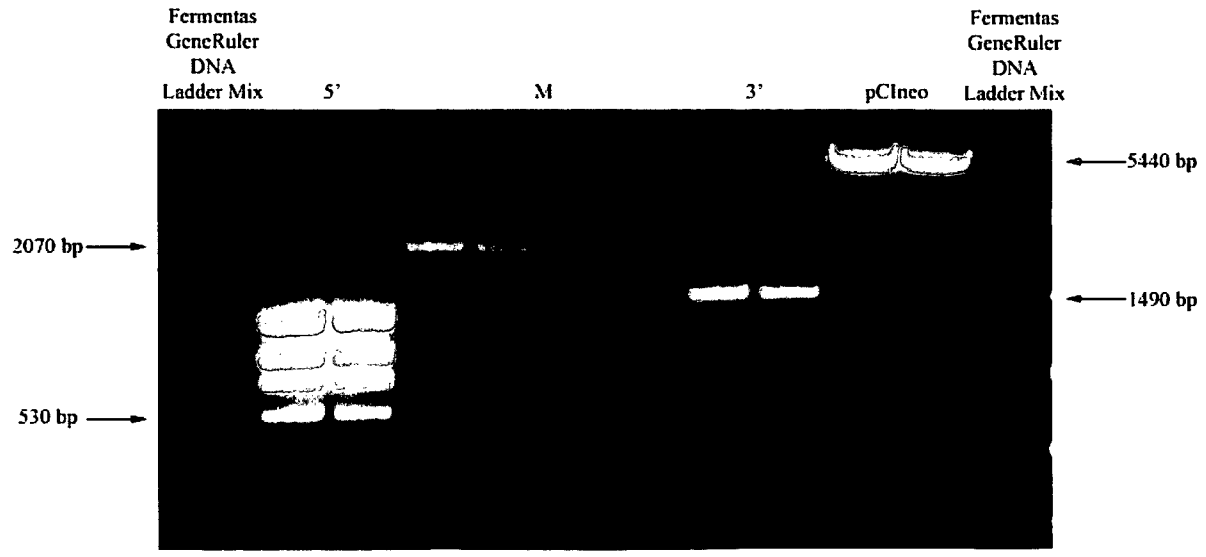

Figure 4.9 – 5′- (lanes 2-3), M- (lanes 4-7), 3′-piece (lanes 8-9) and vector (lanes 10-11) after restriction digestion on 1% TAE-agarose gel.

DNA was extracted from gel and ligation was performed (3.2.1.11) in order to create a circular vector containing all three inserts, as shown in Figure 4.10, **B**. To control proper ligation, 4 µl of the 20 µl ligation mix were analyzed on an agarose gel (Figure 4.10, **A**). Although a small band was visible at about the correct size of ~9500 bp, transformation (3.2.1.12.1) was not successful. No colonies were visible after overnight incubation at 37°C.

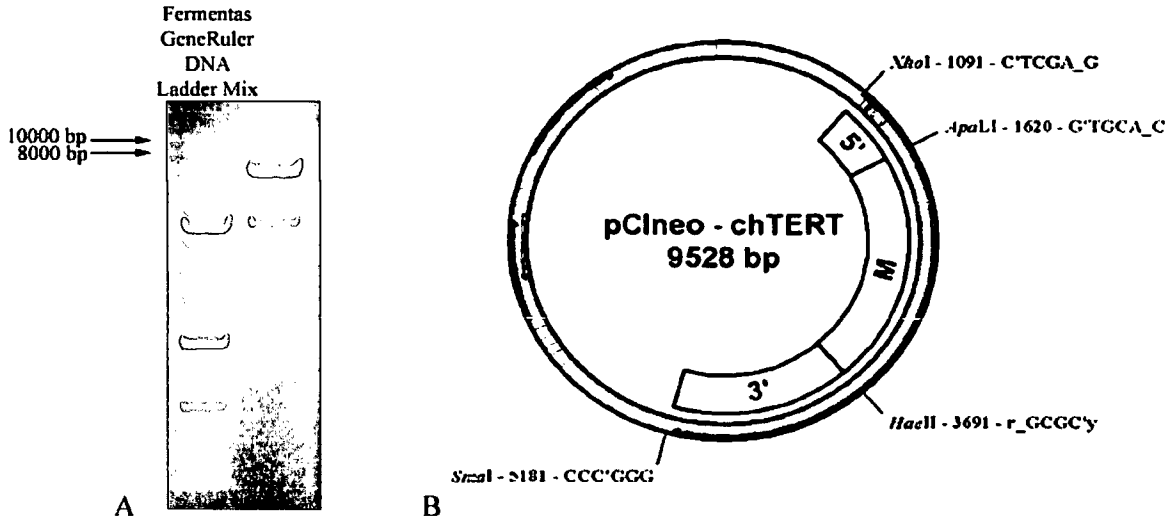

Figure 4.10 - A: Ligation control on 1% TAE-agarose gel. **B:** Vector map of pCIneo containing chTERT gene.

4.2.3. Subcloning of M- and 3′-parts

In order to be able to try out different ligation conditions and not have to perform the reverse transcription of the mRNA and the PCR every time, we decided to ligate the M- and 3′-parts into a vector, so that amplification of each part would be much easier.

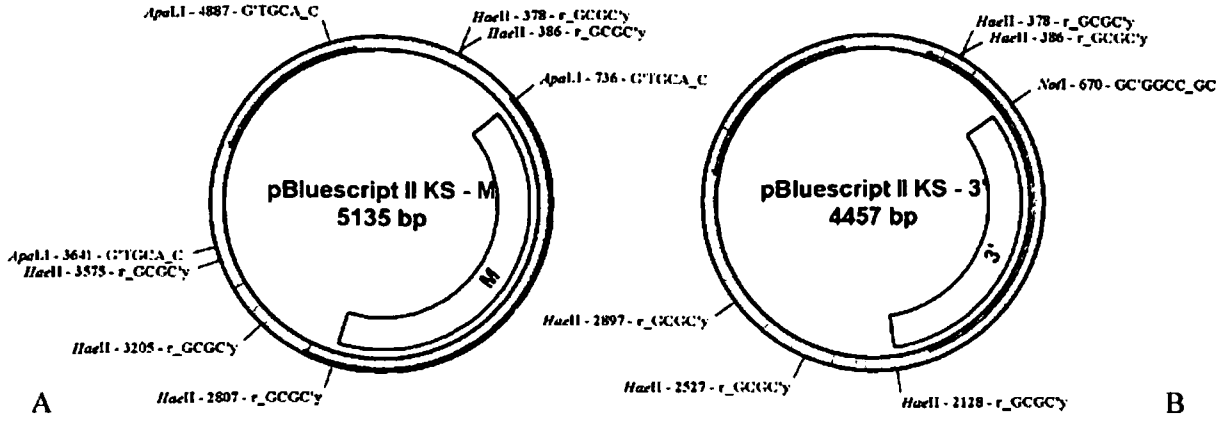

Figure 4.11 - Vector maps of pBluescript II KS with inserted **A**: M-piece. **B**: 3′-piece.

Both parts were again amplified as described in 4.2.1 and blunt-ended. The chosen vector, pBluescript II KS (-), was single cut with SmaI and treated with CIAP (3.2.1.5) to prevent empty recircularization. After ligation and transformation, fifteen colonies (M-part) and five colonies (3′-part) were found on the LB-agar plates containing ampicillin. All colonies were taken for Miniprep plasmid purifications (3.2.1.14.1), which were then digested with HaeII and ApaII (M-part) or HaeII and NotI (3′-part) to identify the correct clones. Since both restriction sites of the M-part are on the insert itself orientation was not significant, whereas, for the 3′-part, the 3′-end of the insert had to be ligated next to the NotI restriction site of the vector. After digestion, fragments of the vector with the M-part should have been ~2070, 1250, 630, 420, 370, 350, 70 and 8 bp big – fragments of the vector with the 3′-part in the correct orientation ~1940, 1490, 400, 370, 300 and 8 bp. As shown in Figure 4.12, clones M/4, M/7 and M/15, as well as 3′/2 and 3′/3 showed the correct fragments.

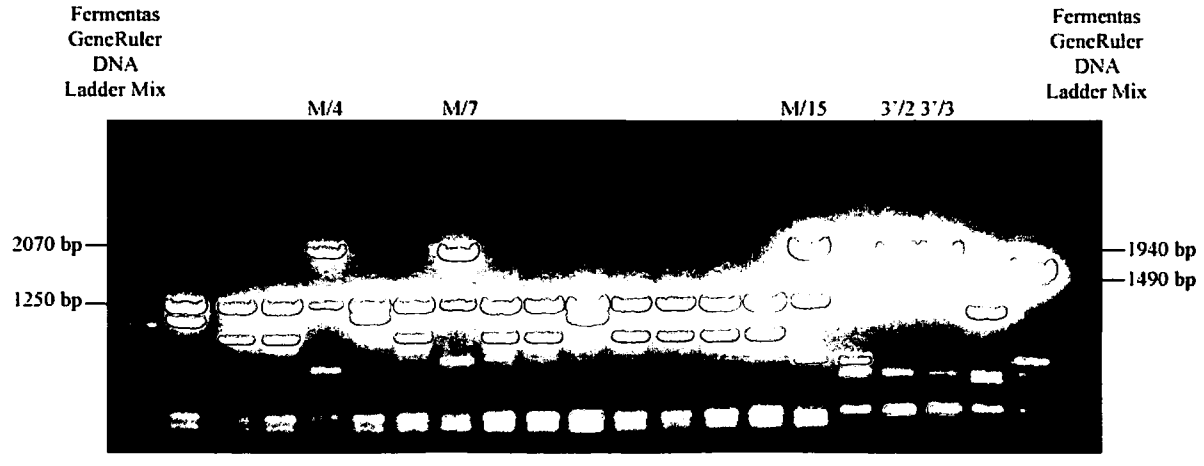

Figure 4.12 - Restriction digest of pBluescript containing M-part (lanes 2-16) or 3′-part (lanes 17-21).

Clones M/15 and 3′/3 were chosen for further investigation. PCRs were performed and results are shown in Figure 4.13. Primers chTERT522sense and chTERT1948AS (desired product at 1426 bp – [1]) as well as chTERT1263sense and chTERT2719AS (product at 1456 bp – [2]) were used to prove the existence of the M-part. chTERT2564sense and chTERT4082AS (product at 1518 bp – [3]) as well as T3promoter and chTERT2719AS (product at 200 bp – [4]) were used for the 3′-part.

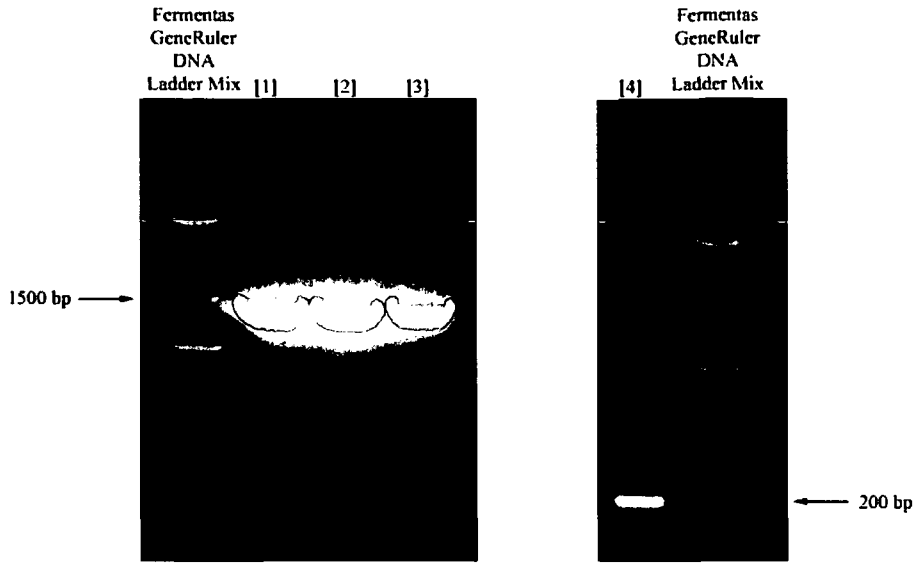

Figure 4.13 - PCR products on 1% TAE-agarose gel – [1] and [2]: specific primers for M-part. [3] and [4]: specific primers for 3′-part.

To double check these results, Midiprep plasmid preparation was performed and both constructs were sequenced by Martin IBL (3.2.1.15). No mutations were found in the 3′-part. The M-part showed some silent mutations and three unknown mutations (change from glutamic acid to glycine at amino acids 292 and 646, and from leucine to serine at amino acid 484). Since we did not think that these mutations would have an effect on the structure or function of the protein, we chose to continue with this construct.

4.2.4. Preparation of plasmid 2

After the successful subcloning additional ligations were performed in order to find out the best way to ligate four parts together. Ideas ranged from ligation of all four parts at the same

time, over ligation of the three insert parts before trying to combine it with the vector, to ligation of only two parts at a time.

Vector and 5′-part were digested just as before (4.2.2) To isolate the M- and 3′-part, the Midipreps from the subcloning (4.2.3) were used instead of PCR products from cDNA. The M-part digestion of the Midiprep was performed in the same way as the one from the PCR product and the 3′-part was digested using HaeII and NotI. The first attempt was to ligate all three insert parts together, cut the correct band (~4100 bp) out of the agarose gel, and ligate it afterwards with the vector. Unfortunately, no band could be isolated at the correct height. As can be seen in Figure 4.14, **A**, the main band is well above 10000 bp, whereas there is only a diffuse smear below. To avoid repeated insert ligation, a second attempt was made to ligate all four parts at the same time because vector circularization would prevent further ligation. The product of this ligation should have been ~9500 bp (Figure 4.10, **B**). Again the main band was well above 10000 bp (Figure 4.14, **B**) and it was not possible to extract a single band for transformation. However, transformation was tried but no colonies were obtained.

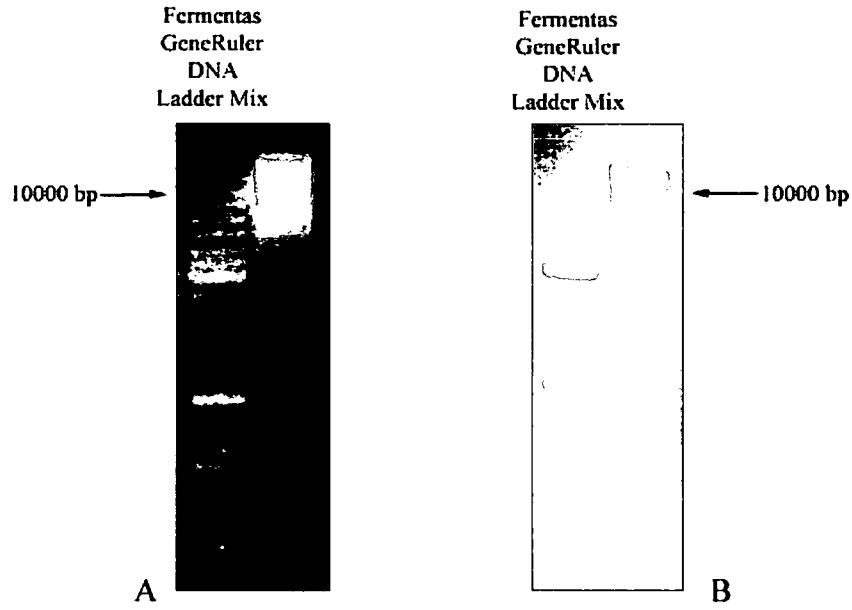

Figure 4.14 - Ligation product on 1% TAE-agarose gel **A**: "Insert ligation". **B:** Ligation of all 4 parts.

After that, we tried to ligate the 5′-part and the M-part, extract the band at ~2700 bp, and ligate it with the band at ~7000 bp after ligation of the vector with the 3′-part. As shown in Figure 4.15, both initial ligations seemed to work, and so both bands were cut from the gel, DNA was extracted and a second ligation was performed. To avoid losing too much DNA, we

did not make a second gel electrophoresis and all of the ligation mix was used for transformation.

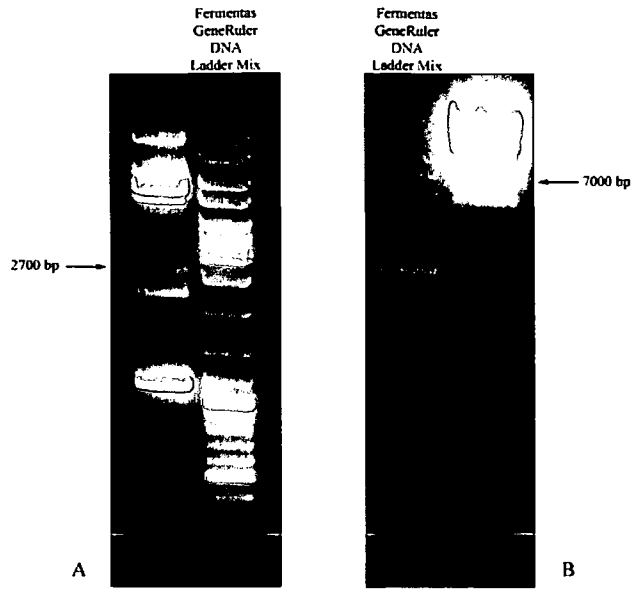

Figure 4.15 - Ligation product on 1% TAE-agarose gel **A:** Ligation of 5´-/M-part. **B:** Ligation of vector/3´-part.

Forty colonies were found on the agar plate after overnight incubation at 37°C. All of them were used to perform Miniprep plasmid preparations, which were then digested with XhoI and NotI. Actually the desired result would have been a vector band at ~5500 bp and an insert band at ~4000 bp but most colonies only showed the vector band or a significantly lower band (Figure 4.16).

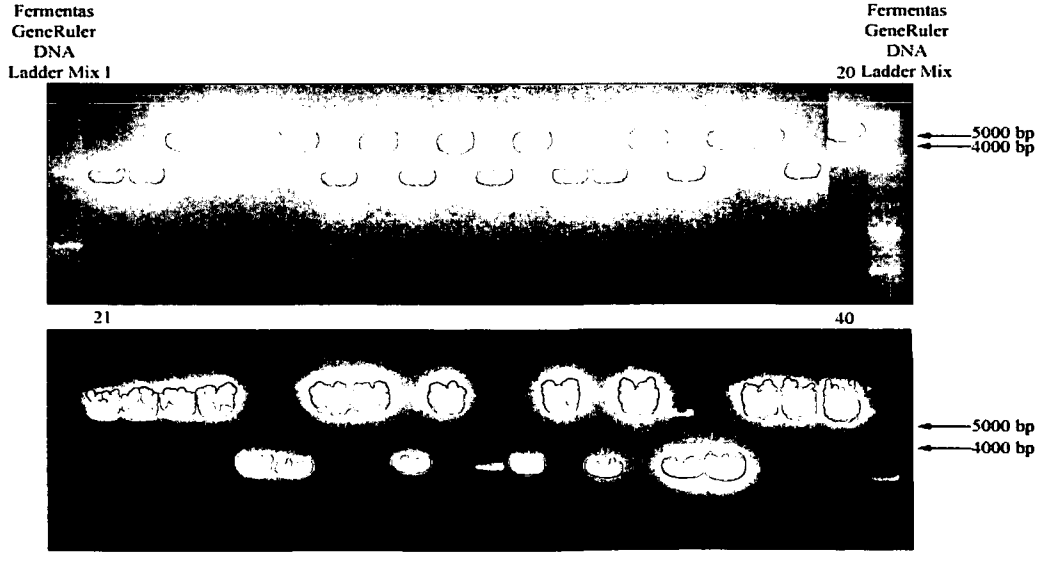

Figure 4.16 - Restriction digestion on 1% TAE-agarose gel - colonies 1-40 after XhoI/NotI digestion.

4.2.5. Subcloning of M3-part

To reduce parts that have to be ligated, we decided to do a second subcloning, transferring the 3´-part into the pBluescript II KS vector already containing the M-part. Since digestion with HaeII and NotI was not possible in order to linearize the vector (Figure 4.11) because HaeII cuts several times within the vector sequence, AfeI was used instead as it only cuts the HaeII recognition site at the end of the M-part and the beginning of the 3´-part (Figure 4.17), and produces blunt ends.

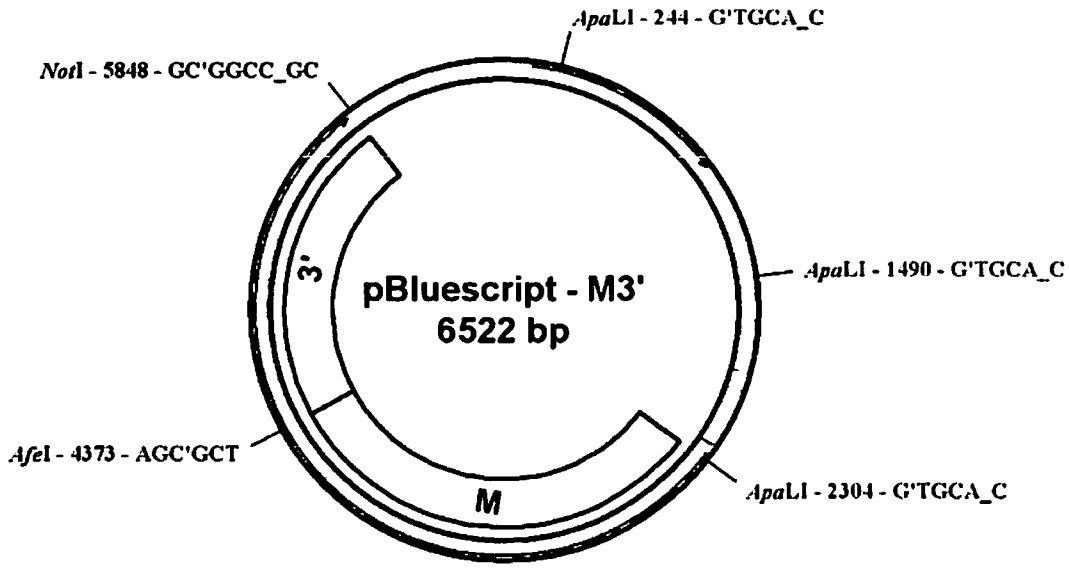

Figure 4.17 - Vector map of pBluescript containing the M- and 3´-parts.

Both vectors, pBluescript – M and pBluescript – 3´, were digested with NotI and AfeI. As seen in Figure 4.18, **A**, there is a clear band of pBluescript – M (clone 15) at ~5000 bp, and two bands of pBluescript – 3´ (clone 3), which are the vector at ~3000 bp and the insert at ~1500 bp. Afterwards, the pBluescript – M-part and the 3´-part were purified and ligated. The ligation product of ~6500 bp is clearly visible in Figure 4.18, **B**, the other band at ~5000 bp is the result of unligated vector.

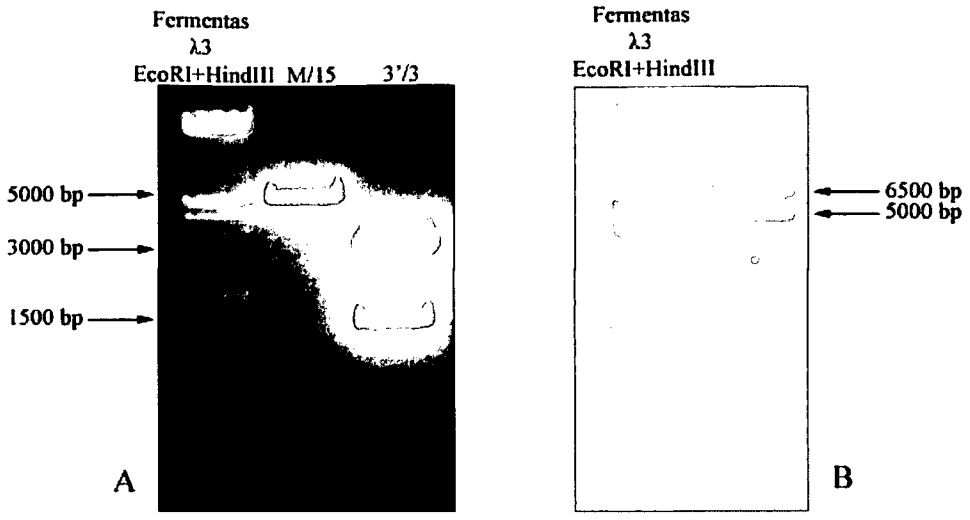

Figure 4.18 - **A:** Restriction digestion of M/15 and 3′/3 with NotI and AfeI. **B**: Ligation of pBluescript - M with 3′-part.

After transformation, five colonies were isolated and Midiprep plasmid purifications were performed. Restriction digestion was performed using NotI and ApaII, which are the flanking restriction sites of the M3-insert, producing fragments of ~3500, 1250, 900 and 800 bp in case of correct ligation. As seen in Figure 4.19, three clones (2, 4 and 5) showed the correct pattern; clone M3/4 was used for plasmid isolation and further work.

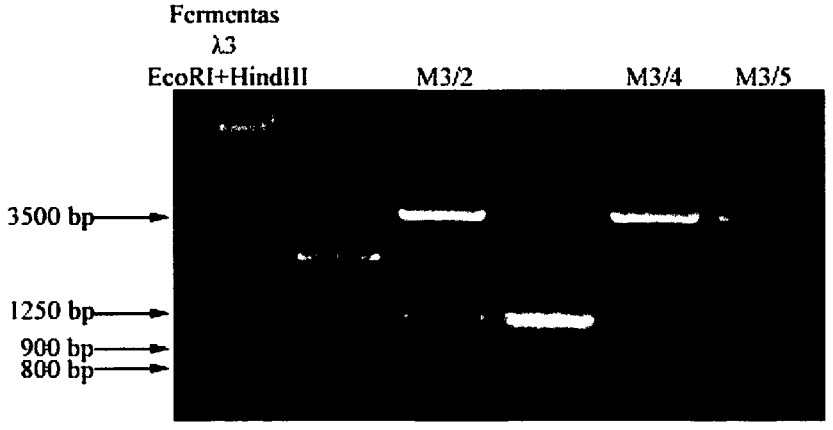

Figure 4.19 - Restriction digestion of pBluescript - M3 constructs using ApaII and NotI.

4.2.6. Preparation of plasmid 3

After successfully subcloning the M3-part, a new attempt was made to create the whole construct (Figure 4.20, **B**). The newly created pBluescript – M3 was digested with ApaII and NotI, the vector pCIneo with XhoI and NotI, the Adriacell-construct containing the 5′-part with ApaII and XhoI, and the desired bands were isolated.

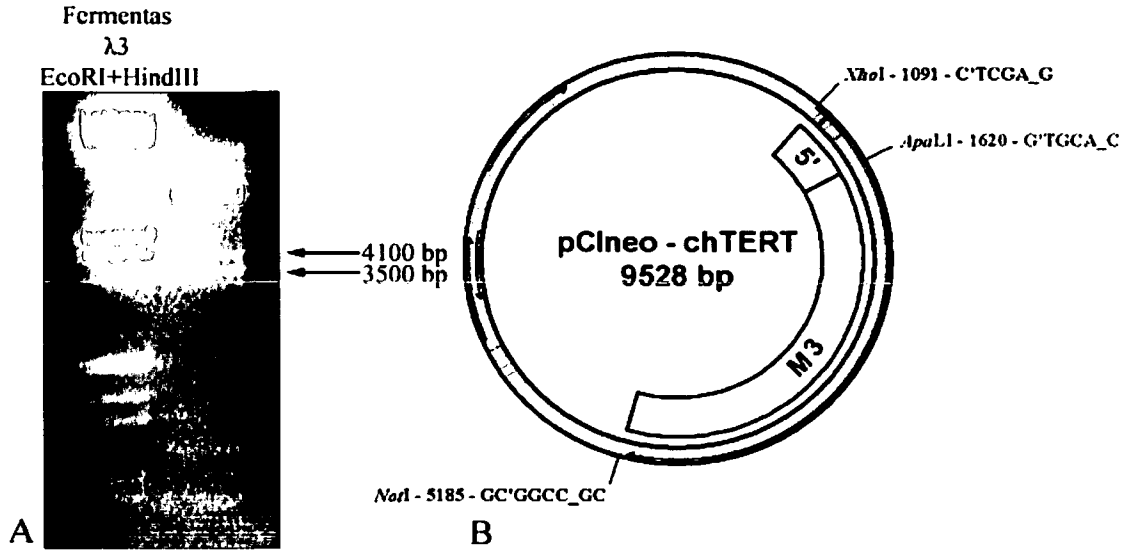

Figure 4.20 - **A:** Ligation product of M3- and 5′-part on 1% TAE-agarose gel. **B:** Vector map of pCIneo – chTERT.

We tried to ligate the M3- and the 5′-part before ligating the whole gene into the vector. The result of the first ligation was a very weak band at the desired size of ~4100 bp. Unligated M3-pieces (~3500 bp) can be seen as well as unknown products at >8000 bp in Figure 4.20, **A**. However, isolation of the ligation product was possible, which was then ligated into the vector. 31 colonies were screened with restriction digestion (HaeII) after Miniprep plasmid purification. Fragments of empty vector should be 8, 1030, 1815 and 2621 bp, whereas vector with correct insert should show bands at 8, 1030, 1900, 2621 and 3953 bp. The band at 3953 bp should contain the complete 5′- and M-part and parts of the vector, and since the M- and 3′-parts were subcloned together this band would have been a proof of correct ligation. Unfortunately, none of the 31 clones showed a pattern that matched either with the empty or the full vector (Figure 4.21). Only three clones (4, 13, 23) had a band at ~4000 bp and even though the other bands were not at the correct height, they were selected for further PCR screening.

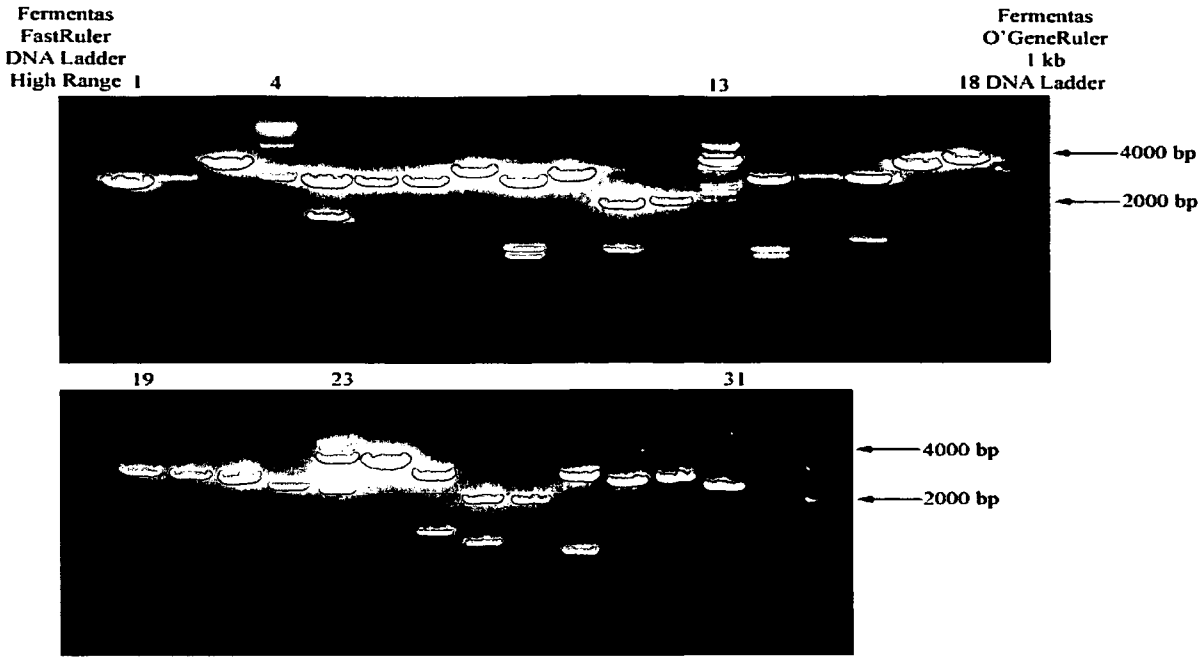

Figure 4.21 - Restriction digestion on 1% TAE-agarose gel – colonies 1-31 after HaeII digestion.

PCR screening was performed as described in 3.2.1.13, using chTERT522sense/chTERT1948AS and chTERT2564sense/chTERT4082AS. The main product of the first PCR, as shown in Figure 4.22, **A**, [1]-[3], is at the correct height of ~1500 bp but there are also a couple of other bands. The second PCR (Figure 4.22, **A**, [4]-[6]) developed bands at <2000 bp, which is quite surprising.

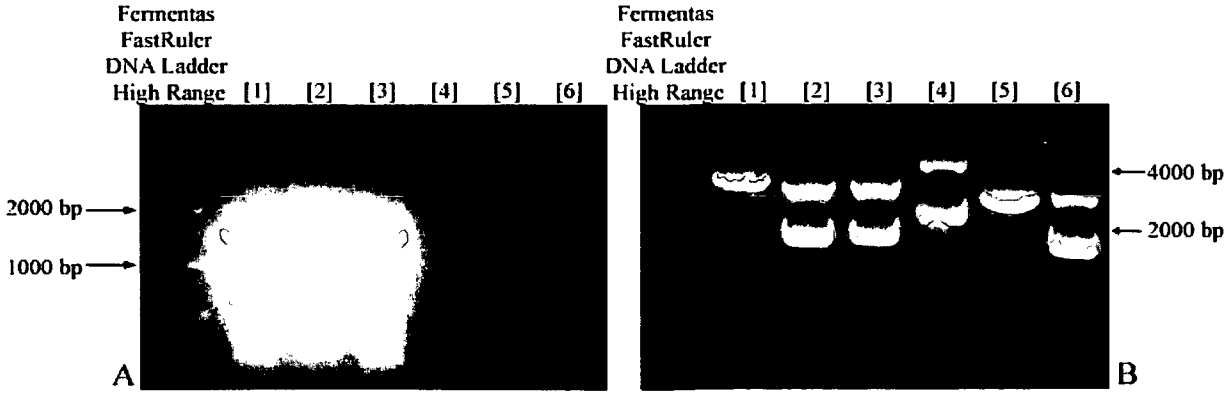

Figure 4.22 - **A:** PCR product on 1% TAE-agarose gel - [1]-[3] chTERT522sense/chTERT1948AS on clones 4, 13 and 23; [4]-[6] chTERT2564sense/chTERT4082AS on clones 4, 13 and 23. **B:** restriction digestion on 1% TAE-agarose gel: [1]-[3] HindIII on clones 4, 13 and 23; [4]-[6] XhoI/NotI on clones 4, 13 and 23.

A restriction digestion was performed to explain the results of the PCR screening. All three clones were digested in a single digest with HindIII (Figure 4.22, **B**, [1]-[3]) and a double digest with XhoI/NotI (Figure 4.22, **B**, [4]-[6]). The single digest should have brought fragments at 849, 2372, 2476 and 3815 bp, the double digest at 4078 and 5434. Any of the restriction patterns showed similarity with neither the empty nor full vector. Another ligation approach to ligate the vector and the M3-part before adding the 5′-part was also tried but it was unsuccessful.

4.2.7. Vector cloning: turning pcDNA3.1 hygro (+) into pcDNA3.1 hygro (-)

Since all ligation/transformation attempts showed unexplainable results, we decided to use another vector instead of pCIneo and chose pcDNA3.1 hygro (-). pcDNA3.1 hygro (+), which has a turned multiple cloning site (MCS), was available in our lab. PmeI, a blunt end cutting enzyme, has restriction sites at the beginning and end of the MCS and can therefore be used to extract nearly the whole MCS, which is then religated with the vector. Depending on the ligation orientation, half of the religated vectors should be (+) oriented while the other half are (-) oriented.

10 µg of pcDNA3.1 hygro (+) were used for digestion with PmeI. Since the fragment of the MCS is only ~100 bp, a 2% TAE-agarose gel was used to separate the ~100 bp MCS fragment and the 5500 bp fragment of the vector without the MCS. The problem is that on one hand we had to use large amounts of vector to get a clear band of the 100 bp fragment, on the other hand we had to take care that no vector stayed undigested because this would have pushed the ratio of (+) to (-) oriented vector after ligation from 50:50 far towards the (+) orientation and would have made screening very time consuming. The undigested and digested vector cannot be separated on a 2% TAE-agarose gel because the difference in size is not big enough. No pictures were taken of this digestion because we tried to keep UV exposure as minimal as possible to save as much as we could of the 100 bp fragment. After gel extraction, the vector was treated with CIAP (3.2.1.5) to prevent self-ligation at the two PmeI-blunt ends. Ligation and transformation were performed. To see if complete digestion and CIAP treatment had worked, the CIAP-treated digested vector was also ligated and transformed. The ideal result would have been no colonies on this plate but >300 colonies indicated that the vector had religated despite the CIAP treatment or had not been completely digested. The plate with the MCS–vector ligation showed an equal amount of colonies. Only

four colonies were chosen to check which of these problems had occurred. Digestion with HindIII and NdeI was chosen to check whether the colonies contained vector without MCS (1982 and 3513 bp), (+) oriented vector (427, 1657 and 3513 bp) or (-) oriented vector (511, 1573 and 3513 bp). Three colonies showed the pattern of (+) oriented vector but the first chosen colony seemed to include the desired (-) oriented vector (Figure 4.7, **A**).

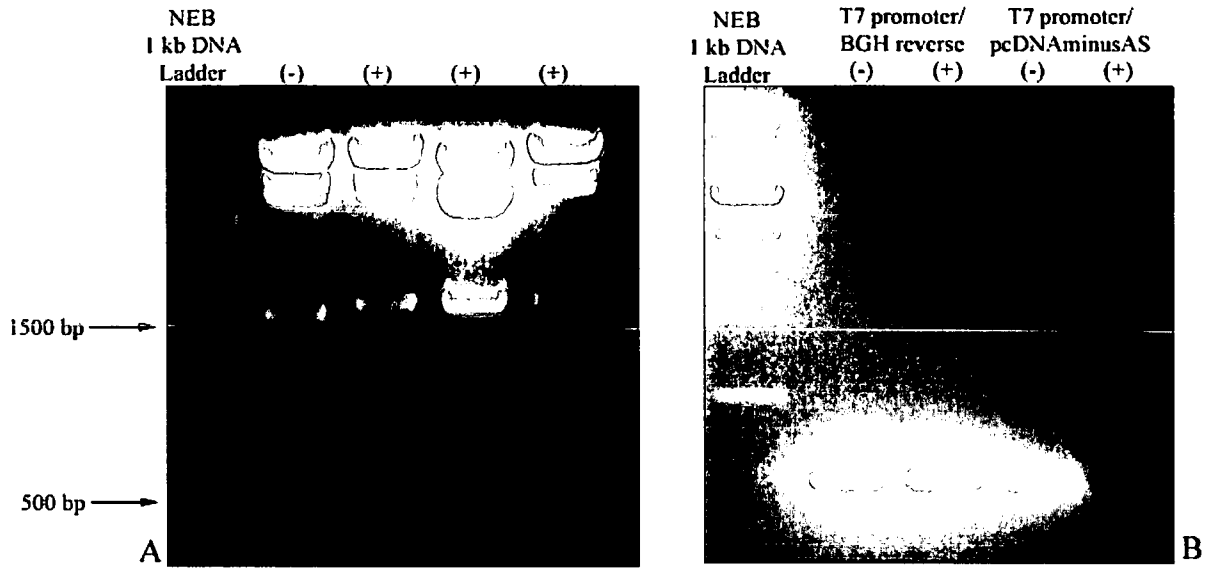

Figure 4.23 – **A:** Restriction digestion on 1% TAE-agarose gel - (-) oriented vector shows slightly different bands at ~500 and 1600 bp. **B:** PCR products on 1% TAE-agarose gel – bands from T7promoter/BGHreverse primer pair at (+) and (-) orientation; band from T7promoter/pcDNAminusAS primers only at (-) oriented vector.

Another digestion of clone 1 and original pcDNA3.1 hygro (+) with HindIII and NdeI also proved this result. Two PCRs were performed as final control, where original pcDNA3.1 hygro (+) was used as control. One primer pair (T7promoter/BGHreverse) was used to check the presence of the MCS, the other (T7promoter/pcDNAminusAS) was used to distinguish between (+) and (-) orientation. pcDNAminusAS was explicitly designed to bind at the PmeI restriction site and the following bases in (-) orientation. The (-) clone was used for a Maxiprep plasmid preparation, which was then sequenced by Martin IBL.

4.2.8. Preparation of plasmid 4

This time we tried again to ligate the M3- and 5′-part before attempting to form the complete vector (Figure 4.24, **B**). Since earlier ligations didn't really work as expected, we wanted to try a couple of different ligation conditions. We gave changes of ligation time, temperature and amount of used enzyme a trial. 10, 15 and 20 minutes of ligation time were tested but no significant difference was observed and for convenience we chose to stay with the ligation at room temperature. The last parameter to change was the amount of enzyme used in the 20 µl reaction mixture (Table 3.6). The original protocol of NEB T4 DNA Ligase suggests the use of 400 U per 20 µl reaction but we tried a reduction to 80 U (0.2 µl). Ligations of M3- and 5′-part were performed and, after incubation, gel electrophoresis was performed. As we had already see, the bands of the 400 U-reaction seemed somehow blurred, whereas the bands of the 80 U-reaction were much more distinctive (Figure 4.24, **A**). This is not only essential for excising single bands from a gel but it is also a cost-saving factor. At ~500 bp the unligated 5′-part and at ~3500 bp the unligated M3-part can be seen. The band at ~1000 bp seems to be a result of self-ligation of the 5′-part. Although it was not the main product, the desired ligation product can be clearly seen at ~4000 bp when only 80 U of ligase have been used.

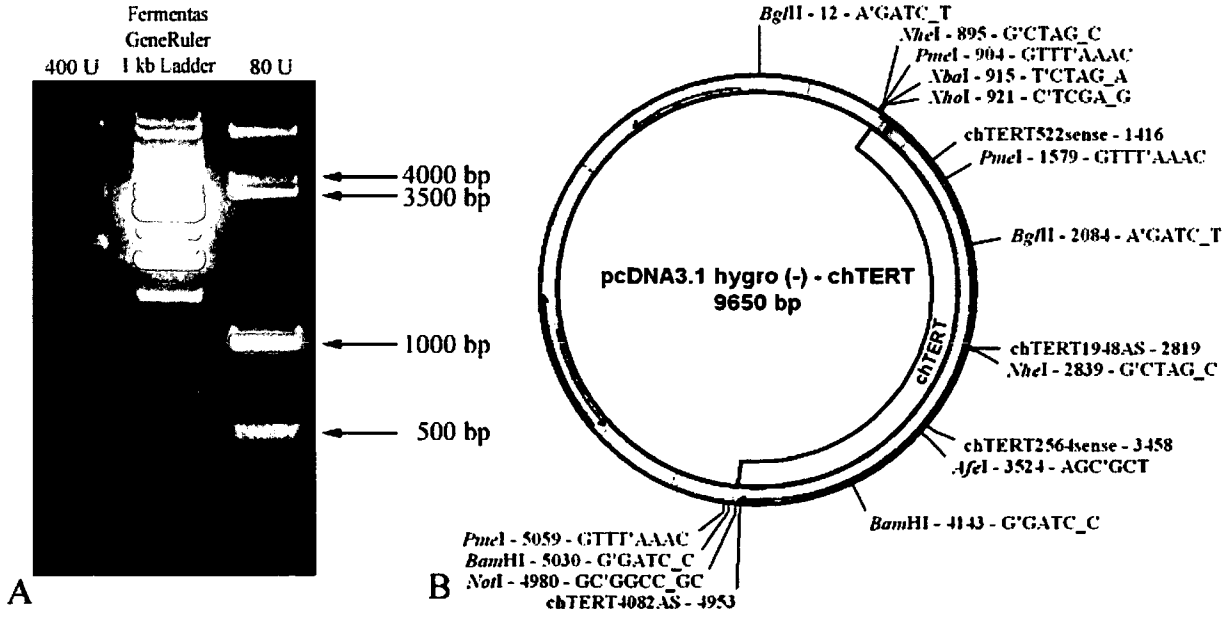

Figure 4.24 - A: Ligation products on 1% TAE-agarose gel with different amounts of T4 DNA Ligase – 400 and 80 units of enzyme. **B:** Vector map of pcDNA3.1 hygro (-) – chTERT.

Therefore we decided to stay with the reduced amount of enzyme for further ligations. Two ligations of the M3- and 5′-part were performed large-scale. 750 ng of 5′-part and 900 ng of M3-part were used in a 60 µl reaction mixture. After gel-electrophoresis ~160 ng of ligation product were isolated in both cases, which were then used for ligation with ~180 ng of digested (XhoI/NotI) and CIAP treated vector. Two separate transformations were performed – in the first case LB-agar plates were incubated at 28°C for 36 hours, in the second at 37°C for 12 hours. On both plates more than 50 colonies were found and PCR screened using the primer pairs chTERT522sense/chTERT1948AS and chTERT2564sense/chTERT4082AS. We started with 19 clones from the plate incubated at 28°C, and obtained strong bands at 1500 bp from all clones using the first primer pair but no bands from the same clones using the second pair (Figure 4.25).

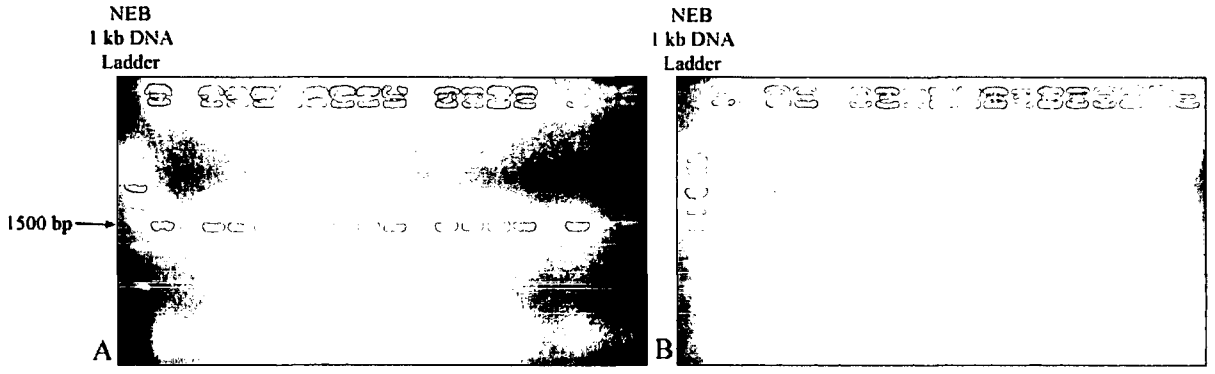

Figure 4.25 - PCR products on 1% TAE-agarose gel. **A:** Clones 1-19 with chTERT522sense/chTERT1948AS. **B:** Clones 1-19 with chTERT2564sense/chTERT4082AS.

So far, this was a strange result as both PCRs only detect the M3-piece, which has not undergone any ligation since successful subcloning and should therefore be completely within the vector. Three additional clones (20, 21 and 22) were tested unsuccessfully with additional primer pairs such as chTERT163sense/chTERT2719AS, chTERT2564sense/BGHreverse and T7promoter/chTERT1110AS. Miniprep plasmid purifications were performed from eight selected clones which showed a band at the PCR screening using the primers chTERT522sense/chTERT1948AS. Double digestion with XhoI/NotI, which should have cut out the whole insert (fragment sizes: 4059 and 5591 bp), and single digestion with PmeI (desired fragment sizes: 675, 3480 and 5495 bp) were carried out.

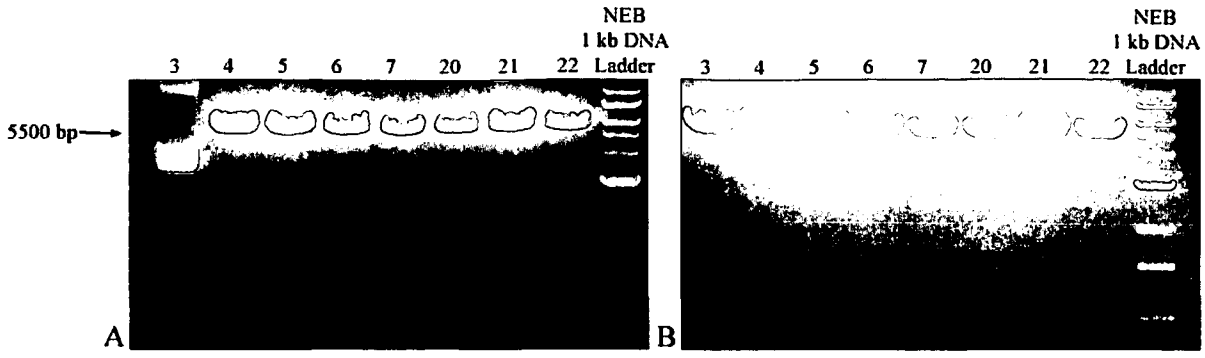

Figure 4.26 - Restriction digestion on 1% TAE-agarose gel. **A:** Double digestion with XhoI/NotI. **B:** Single digestion with PmeI.

None of the screened clones showed the desired restriction pattern but the bands at 5500 bp resembled the pattern of the digested empty vector. The plasmid purifications were used to check the first PCR results (Figure 4.25) and, surprisingly, the main band of the PCR with chTERT522sense/chTERT1948AS had dropped to ~800 bp (left lanes, Figure 4.27). The band at ~1500 bp was very weak compared to the strong and only band of the first PCR. The other PCR with primer pair chTERT2564sense/chTERT4082AS produced no results (right lanes, Figure 4.27).

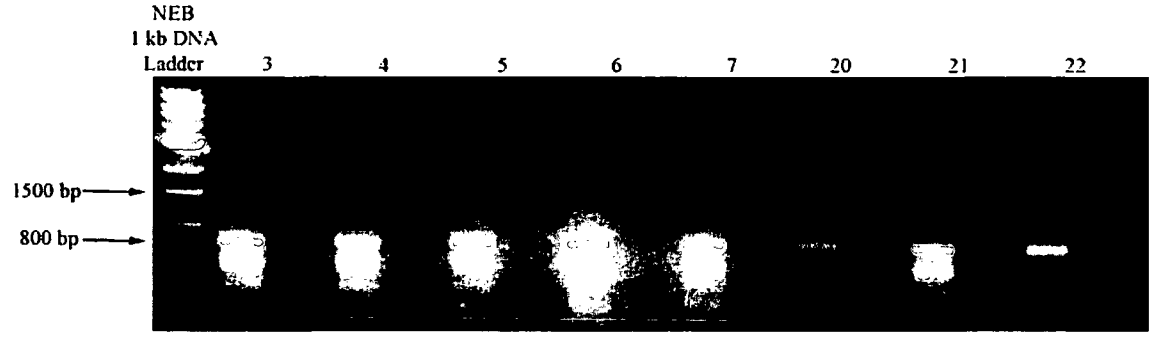

Figure 4.27 - PCR product on 1% TAE-agarose gel - left lanes: chTERT522sense/chTERT1948AS, right lanes: chTERT2564sense/chTERT4082AS.

To gain better insights into these results, four different enzymes (BamHI, BglII, NheI and XbaI) were chosen for single digestion of plasmid purification of clones 21 and 22 (Figure 4.28). Both plasmids were also applied without enzyme to see if digestion worked. As seen in Figure 4.24, **B**, XbaI should not cut within the insert but only once within the vector – the found band is significantly lower than the expected 9560 bp. NheI was expected to cut twice,

once within the insert at ~2000 bp and once within the MCS before the insert. BglII should have cut at ~1200 bp within the insert and within the vector sequence, and finally BamHI should have cut at ~3200 bp within the insert and within the MCS after the insert. No enzyme led us to more than one band. This suggests that the vector was empty, which is again disproved by PCR results.

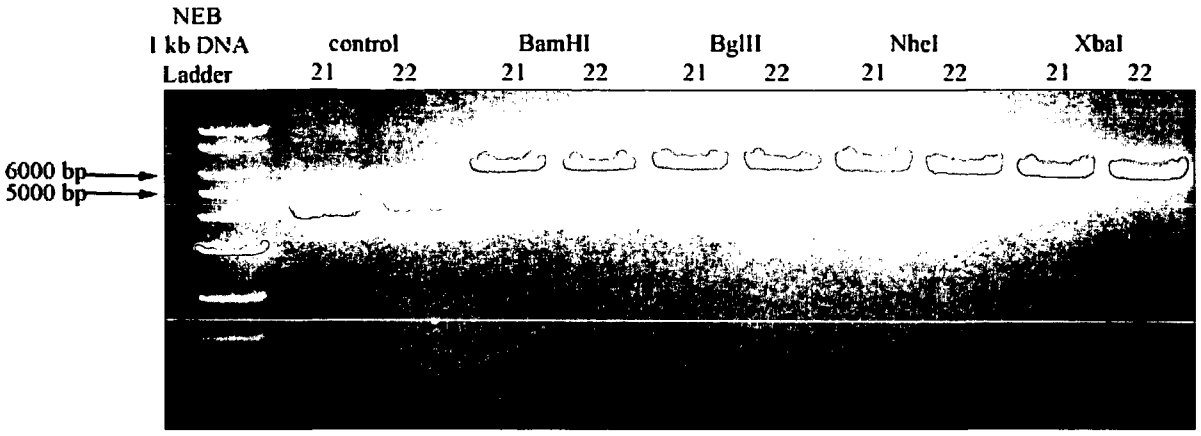

Figure 4.28 - Restriction digestion on 1% TAE-agarose gel - clones 21 and 22 without enzyme and with BamHI, BglII, NheI and XbaI.

In total, 60 colonies were PCR screened with chTERT2564sense/chTERT4082AS but no bands were detected. Colonies from the plate incubated at 28°C were also screened without positive results.

4.3. Generation of genetic constructs: exchange of antibiotic-resistance genes

After unsuccessful chTERT-cloning, we tested our modus operandi at another cloning experiment. The presetting of this work was to take an insert containing vector with ampicillin and hygromycin resistance genes and substitute these genes for a kanamycin resistance gene. This was necessary since a cooperation partner of our working group wanted to use this vector for some experiments but company policies did not allow the use of ampicillin. The vector was pSilencer 2.1-U6 hygro (Ambion) containing three different inserts: SNEV shRNA, luc1 shRNA and pre-hsa-miR193a. The inserts were all about 100 bp in size, and positioned within the HindIII- and BamHI-restriction sites. As seen in Figure 4.29, **A**, the origin of replication,

the U6 promoter and the insert are positioned within the restriction sites of SspI and AhdI. A double digestion of the vector with these two enzymes brought bands at 804, 1755 and ~2400bp (exact size depending on insert). The largest band was the desired band, which contained all important genetic information without antibiotic-resistance genes.

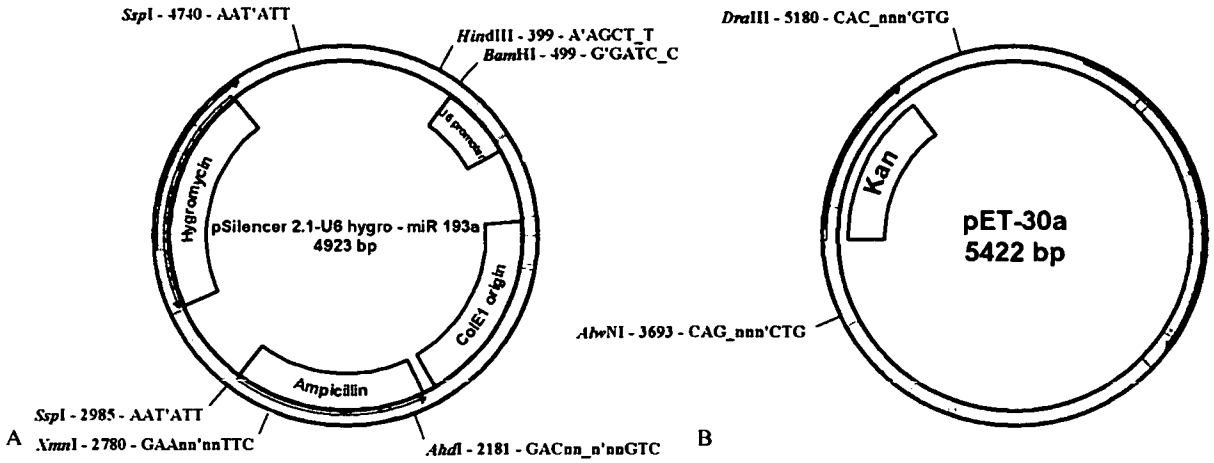

Figure 4.29 – A: Vector map of pSilencer 2.1-U6 hygro - miR193a. **B:** Vector map of pET30a.

Our original idea was to use the Kan^R/Neo^R-cassette out of the vector pEGFP-N1 (Clontech) after digestion with DraIII and EcoO109I. The ~2000 bp fragment was extracted and ligated with the ~2400 bp pSilencer-fragment but no colonies were found after transformation (data not shown here). Since neomycin resistance was not really necessary for the experiment, we decided to change the donating vector and take only the kanamycin resistance gene out of pET30a (Kan^R/pET30a). Digestion with DraIII and AlwNI was performed, and the ~1500 bp fragment including the kanamycin resistance gene was extracted (Figure 4.29, **B**). Owing to the incompatible sticky ends, blunt ends were produced to allow ligation. Between 100 and 150 ng of digested pSilencer containing the different inserts without resistance genes (~2400 bp) were ligated with 230 ng of Kan^R/pET30a (~1500 bp), which is a vector/insert ratio of 1:2.5 – 1:4, and used for transformation after half of each reaction mix had been applied to a 1% TAE-agarose gel.

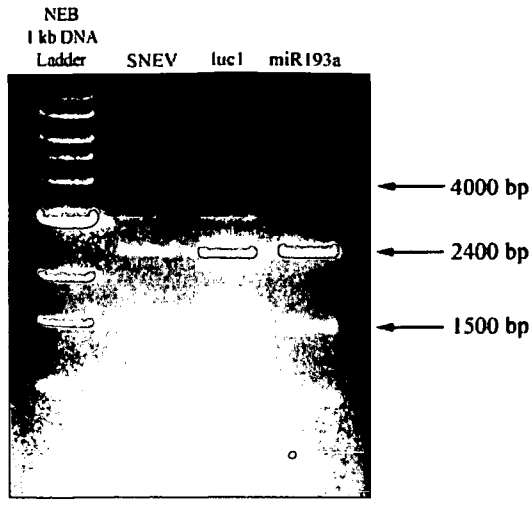

Figure 4.30 - Ligation products on 1% TAE-agarose gel.

The gel showed clear bands of unligated pSilencer (~2400 bp), unligated Kan^R/pET30a (~1500 bp) and the desired ligation product at ~4000 bp (Figure 4.30). Ten colonies containing pSilencer 2.1-U6 **kan** – SNEV, 126 colonies containing pSilencer 2.1-U6 **kan** – luc1 and 136 colonies containing pSilencer 2.1-U6 **kan** – miR193a were found after incubation at 28°C for 36 hours. Ten clones of each transformation were used for a Miniprep plasmid preparation. To check the correct size of the vector, single digestions with XmnI were carried out.

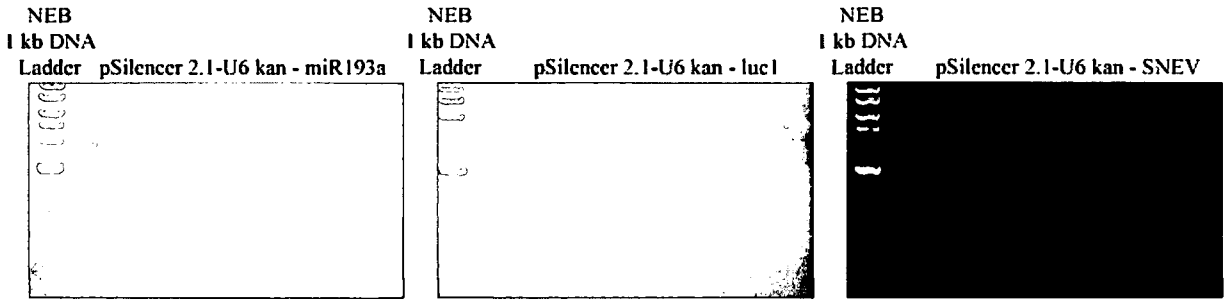

Figure 4.31 - Restriction digestions with XmnI of 10 clones from each of the three genetic constructs on 1% TAE-agarose gel.

As seen in Figure 4.31, nine clones of pSilencer 2.1-U6 **kan**– miR193a, nine clones of pSilencer 2.1-U6 **kan** – luc1 and five clones of pSilencer 2.1-U6 **kan** – SNEV showed a band at the correct height of ~4000 bp.

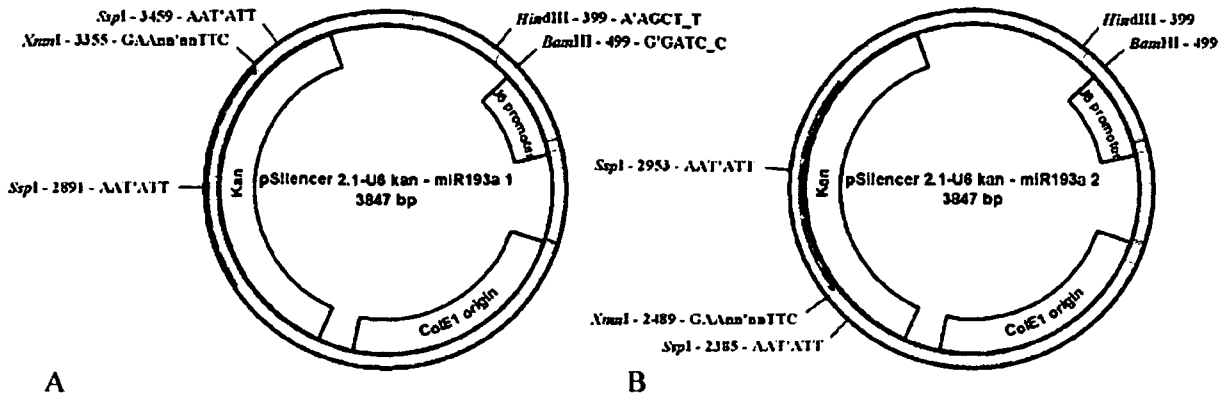

Figure 4.32 - Vector maps of pSilencer 2.1-U6 **kan** – miR193a. **A:** Orientation 1. **B:** Orientation 2.

Owing to the blunt end ligation, there were two possible ways to form a stable construct, depending on how the Kan^R/pET30a-part ligated into the pSilencer vector. Since this part has its own promoter, both orientations should work well, but although we determined the orientation of clones by double digestion with BamHI and XmnI. Orientation 1 (Figure 4.31, **A**) showed fragments of 991 and 2856 bp; orientation 2 (Figure 4.31, **B**) showed 1857 and 1990 bp. Exact fragments lengths of pSilencer 2.1-U6 **kan** – miR193a; luc1- and SNEV-constructs were slightly different.

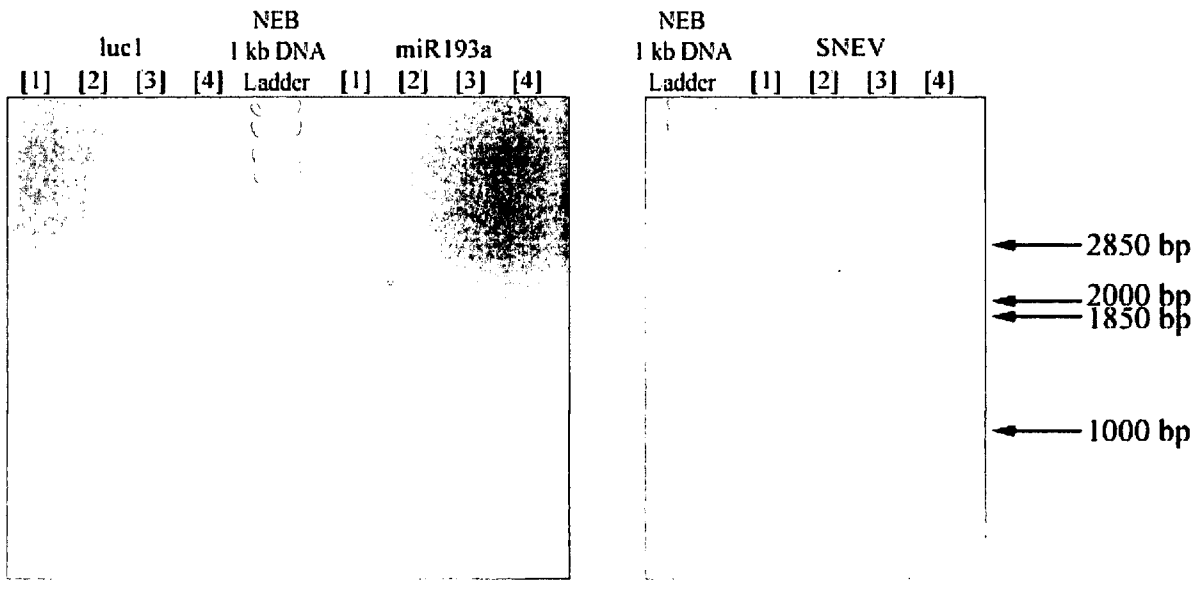

Figure 4.33 - Restriction digestion with BamHI and XmnI on 1% TAE-agarose gel: four clones of each genetic construct.

Four clones were chosen and double-digested. Clone [2] of pSilencer 2.1-U6 **kan** – miR193a and clone [3] of pSilencer 2.1-U6 **kan** – SNEV showed the fragment pattern of orientation 1, all other clones showed orientation 2 (Figure 4.33).

4.4. Generation of genetic constructs: transfer of four different miRNA inserts from pSilencer 2.1-U6 hygro into pcDNA3.1 hygro (-)

After the successful generation of pcDNA3.1 hygro (-), we decided to use this vector for another transfection experiment. Four different miRNA sequences (pre-hsa-miR21, pre-hsa-miR29a, pre-hsa-miR31 and pre-hsa-miR193a) had to be excised from pSilencer 2.1-U6 and transferred to pcDNA3.1 hygro (-). The sequences were flanked by the restriction sites of BamHI and HindIII, which were chosen to extract the inserts (~100 bp). Vector and inserts were separated by agarose gel electrophoresis. To avoid damages and degradation of the short DNA fragments, we tried to keep UV exposure as minimal as possible and did not photograph the gel. The new vector, pcDNA3.1 hygro (-) was digested in the same way and CIAP treated to prevent re-ligation. Then 75 ng of inserts (~100 bp) were ligated with 180 ng of vector (~5500 bp), which corresponds a vector/insert ratio of approximately 1:20. As described before, we used only 80 U of T4 DNA ligase per reaction mixture. Transformation and overnight incubation at 37°C brought more than 200 colonies per plate. PCR screening of 4 colonies per construct was performed with primer pair T7promoter and BGH reverse. All screened colonies showed the desired PCR product at ~250 bp, which suggested the successful ligation of the different inserts with pcDNA3.1 hygro (-). This result was later on confirmed by sequencing.

5. Discussion

The major results that were obtained from cell culture experiments were quite like expected. The primary CEF cells reached PD26 before becoming senescent and stopping proliferation. This is in accordance to previous publications (Hay, 1970). Although viral oncogenes of SV40 did not lead to an immortalization, the positive transfection and expression of viral proteins was clearly verifiable. Staining of viral proteins with fluorescent stains was successful and detected via confocal microscopy and flow cytometry. Although the strong variations in the percentage of transfected cells detected by flow cytometry from measurement to measurement are somehow unclear. The plasmid used for the transfection was the same as used for the transfection and successful immortalization of human fibroma cells earlier in our group (Voglauer et al., 2005). Murine cells are also reported to be immortalized by using this strategy (Tevethia et al., 1998). However, different studies of human fibroblasts state that transfected cells have an increased life-span but do not immortalize (Neufeld et al., 1987; Shay and Wright, 1987). Nevertheless, all studies mention dramatic changes in morphology of transfected cells. Since the ability of SV40 to interact with the growth supressors p53 and pRB, which can hold the cell cycle at G_1/S position, initiate apoptosis or activate DNA repair proteins, is crucial to avoid a cell cycle arrest, structural differences between p53 in human and chicken cells could have caused the obvious absence of SV40/p53 interaction (Greider, 1996; Sherr and DePinho, 2000)

Cloning work for chTERT immortalization was started from a RNA isolation of DT40 cells, which are known to express high levels of chTERT (Swanberg and Delany, 2005). RNA was reversely transcriped into cDNA. According to the published sequence of chTERT (NM_001031007, NCBI reference sequence) different primer pairs were used to amplify the whole protein coding sequence (~4100 bp). Unfortunately my predecessors found out that a PCR of the beginning of the sequence was not possible with all kinds of primers. It was experienced that the first 500 bp of the chTERT sequence (5′-part) are very GC-rich and therefore pose a problem for PCR. To circumvent PCR of this part, the sequence was artificially synthesized. To ease PCR of the remaining part, we decided to amplify two shorter regions (M-part: ~2100 bp; 3′-part: ~1600 bp), which are ligated afterwards with the vector and the 5′-part. During the whole procedure, ligation seemed to be the critical point because 3 parts of insert had to ligate with the vector to form a rather big (~9500 bp) circular plasmid.

After not getting successfully transformed cells, we decided to sub-clone the M- and 3′-part. The PCR, digestion and ligation into pBluescript II KS worked fine, which was approved by sequencing of the vectors. Although this helped us around doing reverse transcription of RNA and PCR of cDNA, it did not improve ligation conditions because we still had four parts to ligate. So we did a second sub-cloning step and brought M- and 3′-part together into pBluescript II KS (M3-part). So far, we did not have any problems with vector recombination after introducing chTERT sequence. However, when we ligated the 5′- and M3-part into pCIneo, we got unexplainable results after restriction digestion which we put down to vector recombination. Another vector, pcDNA3.1 hygro (-), was used for the next ligation attempts since we reasoned that a different vector might help to circumvent the problem. The correct ligation of 5′- and M3-part was proved by gel electrophoresis. The band of the ligation product was excised and used for the ligation with the vector. After transformation, we used two primer pairs to search for correct clones. The first primer pair (chTERT522sense/chTERT2719AS) had been used to amplify the M-part from the cDNA, the second pair (chTERT2564sense/chTERT4082AS) had been used to amplify the 3′-part. Surprisingly the first primer pair led to bands at the desired height at most clones, while the second primer pair did not produce any. But since M- and 3′-part had already been sub-cloned together, this was not really a constructive result because it again implicated vector recombination after bringing 5′- and M3-part together. Restriction analysis showed that the ligated vector had a size of about 5500 bp, which is approximately the size of an empty vector but of course an empty vector would not have led to bands with chTERT specific primers at the absolutely correct height. This predicament brought us to stop further cloning attempts. Therefore the initial aim to transfect CEF cells with the chTERT gene and generating an immortalized cell line was not accomplished.

Fortunately, the correct performance of general cloning methods like restriction digestion, ligation and transformation was approved by three other cloning experiments. The turning of the MCS from pcDNA3.1 hygro (+) to form pcDNA3.1 hygro (-) and the transfer of various miRNA sequences from pSilencer 2.1-U6 hygro into pcDNA3.1 hygro (-) worked satisfactorily after changing some ligation parameters, especially the amount of used ligase, and carefully minimizing the amount of UV exposure. The exchange of antibiotic resistance genes from pSilencer 2.1-U6 worked fine as well after finding a suitable donor for the kanamycin resistance gene (pET30a). The two possible orientations of the kanamycin resistance gene were both found in transformed cells as expected.

Since we found out that general cloning methods work well in our hands, we were puzzled about the difficulties we had at cloning the published variant of the chTERT gene (Delany and Daniels, 2004). Our problems were mainly focused on the first 500 bp. This is the region where the biggest sequence difference between human and chicken TERT is located (Figure 5.1). While exons 3-16 are identical or just show a slight difference, there are vast varieties in exons 1 and 2.

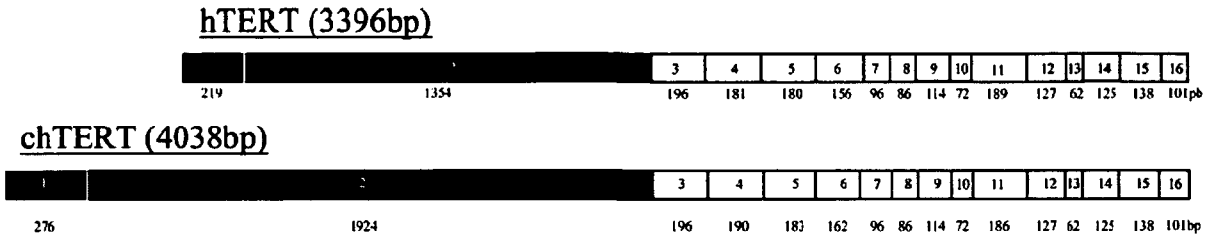

Figure 5.1 - Differences between hTERT and chTERT genes. The exons of identical length in published chTERT and hTERT genes are shown in gray, exons slightly different in size (by 3–9 bp) are hatched, and exons differing significantly in length are shown in black (Fragnet et al., 2005).

This and the fact that lateron during our work nineteen chTERT splicing variants were published (Chang and Delany, 2006) casts doubts on the 5′-piece we used for our cloning work. Figure 4.8 shows that we also had different amplicons after PCR of M- and 3′-part. Especially for the M-part, the 2200 bp amplicon we chose to use for subcloning is not the quantitatively biggest band.

This inconsistency suggest that proper verification of the published sequence in view of splicing variants should preceed further work on this subject. Sythesizing of the whole gene using codon optimization could be helpful to establish an immortalized cell line. This should minimize the recombination potential and alternate splicing while bringing maximum yield of telomerase. Still it has to be paid attention to work with the one splicing variant, which is the biological active form.

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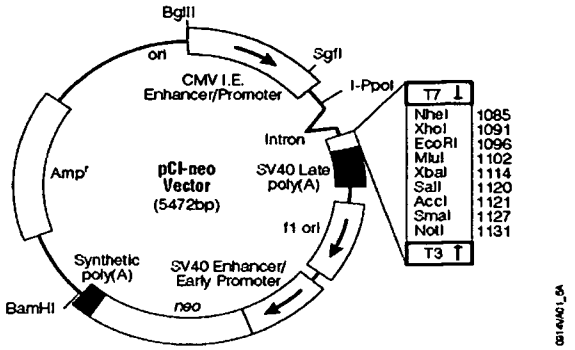
7. Appendix

7.1. Original vector maps

7.1.1. pCIneo (Promega)

Promega

Figure 2. pCI-neo Vector circle map and sequence reference points. Neo = neomycin phosphotransferase; CMV I.E. = cytomegalovirus immediate-early.

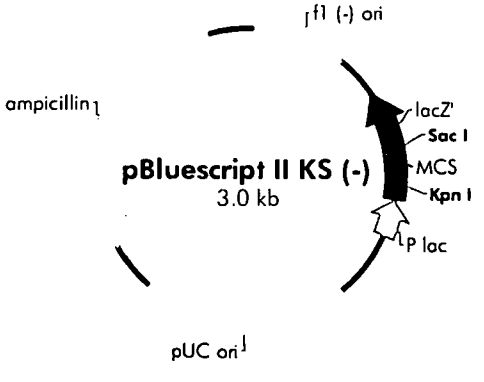


pCI-neo Sequence Reference Points:

CMV immediate-early enhancer/promoter region	1–750
Chimeric intron	890–1022
T7-EEV sequencing primer binding region	1053–1074
T7 RNA polymerase promoter (-17 to +2)	1067–1085
Multiple cloning region	1085–1137
T3 RNA polymerase promoter (-17 to +3)	1140–1158
SV40 late polyadenylation signal	1167–1388
Phage f1 region	1483–1938
SV40 enhancer and early promoter	2000–2418
SV40 minimum origin of replication	2316–2381
Coding region of neomycin phosphotransferase	2463–3257
Synthetic polyadenylation signal	3321–3369
β-lactamase (Amp^r) coding region	3780–4640

7.1.2. pBluescript II KS (-) (Stratagene)

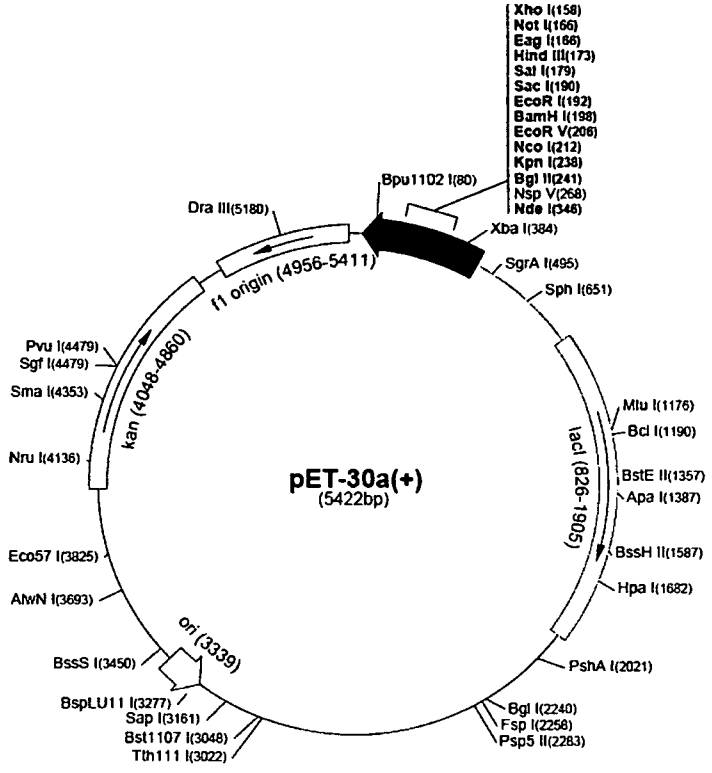
f1 (–) origin 21–327
β-galactosidase α-fragment 460–816
multiple cloning site 653–760
***lac* promoter** 817–938
pUC origin 1158–1825
ampicillin resistance (*bla*) ORF 1976–2833



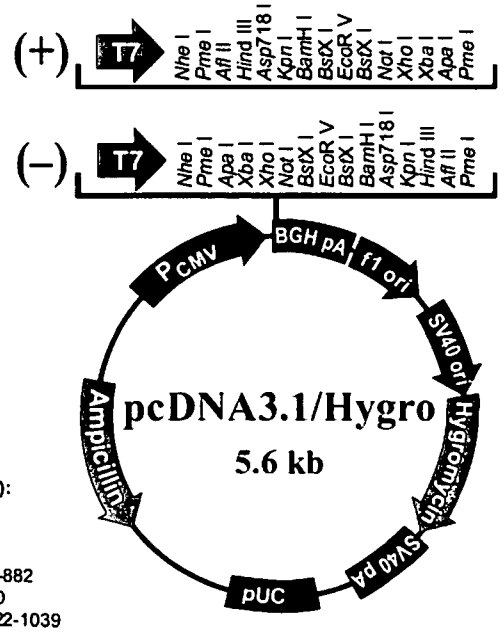
7.1.3. pET30a (+) (Novagen)

pET-30a(+) sequence landmarks	
T7 promoter	419-435
T7 transcription start	418
His•Tag coding sequence	327-344
S•Tag coding sequence	249-293
Multiple cloning sites	
(*Nco* I - *Xho* I)	158-217
His•Tag coding sequence	140-157
T7 terminator	26-72
lacI coding sequence	826-1905
pBR322 origin	3339
Kan coding sequence	4048-4860
f1 origin	4956-5411

The maps for pET-30b(+) and pET-30c(+) are the same as pET-30a(+) (shown) with the following exceptions: pET-30b(+) is a 5421bp plasmid: subtract 1bp from each site beyond *Bam*H I at 198. pET-30c(+) is a 5423bp plasmid: add 1bp to each site beyond *Bam*H I at 198.



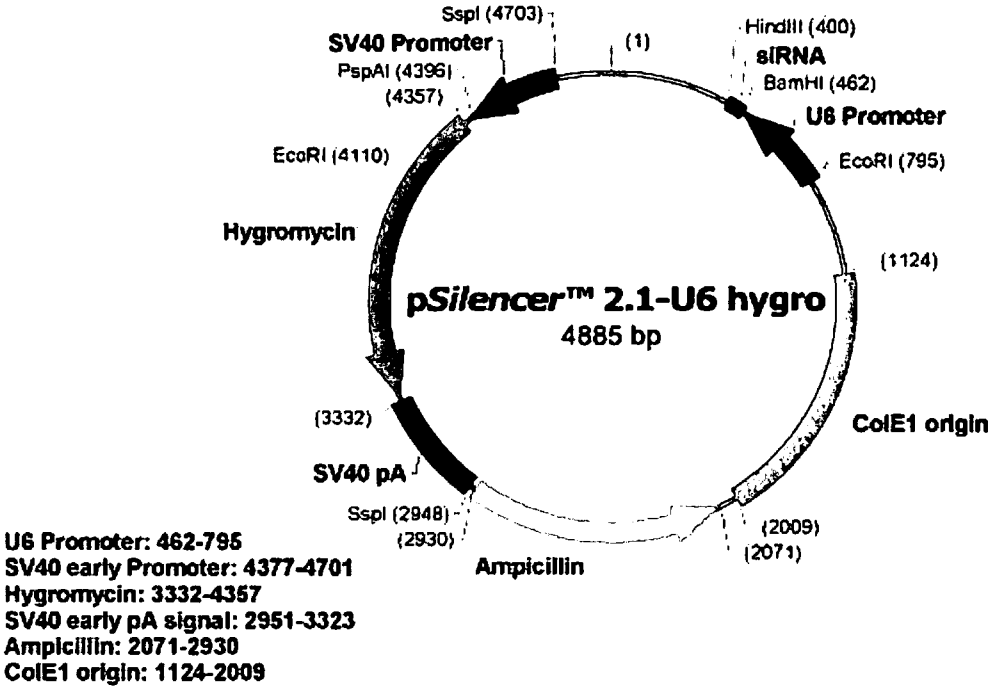
7.1.4. pcDNA3.1 hygro (-) (Invitrogen)



Comments for pcDNA3.1/Hygro (+):
5597 nucleotides

CMV promoter: bases 209-863
T7 promoter/priming site: bases 863-882
Multiple cloning site: bases 895-1010
BGH reverse priming site: bases 1022-1039
BGH polyadenylation signal: bases 1021-1235
f1 origin: bases 1298-1711
SV40 promoter and origin: bases 1776-2100
Hygromycin resistance gene: bases 2118-3141
SV40 early polyadenylation signal: bases 3154-3526
pUC origin: bases 3786-4456 (complementary strand)
Ampicillin resistance gene: bases 4601-5461 (complementary strand)

7.1.5. pSilencer 2.1-U6 hygro (Ambion)



7.2. Primer sequences

T3 promoter	ATTAACCCTCACTAAAGGGA
T7 promoter	TAATACGACTCACTATAGGG
BGH reverse	TAGAAGGCACAGTCGAGG
chTERT522sense	GGATAGGGGACGACGTGATGATGT
chTERT1263sense	TAAAGACCGGTGCAGAAAAACGAG
chTERT2564sense	GTAAGACTAAGCCGTGTTGTTG
chTERT2719AS	CCCGAATACTGAAGAGCCAATGAT
chTERT4082AS	TCCCTTAGTCCAGTATAGTTTTGA

7.3. List of abbreviations

AD	aqua destilata
Amp	ampicillin
AS	antisense
ATP	adenosin triphosphate
bp	base pairs
BSA	bovine serum albumine
CIAP	calf intestinal alkaline phosphatase
CEF	chicken embryo fibroblasts
cDNA	complementary DNA
chTERT	chicken telomerase reverse transcriptase
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide
ddNTP	di- deoxynucleotide
DSB	double strand break
dsDNA	double strand DNA
E. coli	Escherichia coli
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
G 418	geneticin sulphate
hTERT	human telomerase reverse transcriptase
hygro	hygromycin
Kan	kanamycin
LA 2000	Lipofectamine 2000
LB	Luria bertani
MCS	multiple cloning site
mRNA	messenger RNA
miRNA	micro RNA
NaOH	sodium hydroxide
OD	optical density
ORF	open reading frame

ORI	origin of replication
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PD	population doubling
PDL	population doubling level
pT	post transfection
RNA	ribonucleic acid
SPF	apecific pathogen-free
SV40	simian virus 40
TAE	Tris-acetate EDTA
TR	telomerase RNA
TERT	telomerase reverse transcriptase
TRF	telomere repeat-binding factor
UV	ultraviolet