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ANALYSE DES MODIFICATIONS DE LA CHROMATINE IMPLIQUEES DANS L'EFFET BARRIERE DE CTF1 AUX TELOMERES HUMAINS

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SUMMARY

Eukaryotic genomes are compartmentalized in different structural domains that can affect positively or negatively gene expression. These regions of euchromatin and heterochromatin are characterized by distinct histones marks which can facilitate or repress gene transcription. The chromatin environment represents thus one of the main problems to control gene expression in biotechnological applications or gene therapy, since its expression is affected by the chromatin neighboring its *locus* of insertion. Some chromatin regions like telomeres are composed of constitutive heterochromatin which leads to the telomeric position effect (TPE) that silences genes adjacent to the telomere. TPE is known to spread by the self-recruitment of the SIR histone deacetylase complex from the telomere in *S.cerevisiae,* but the histone marks that are associated to telomeric chromatin in mammalian cells remain mostly unknown.

The transcription factor CTF1 has shown antisilencing properties in mammalian cells and also a boundary activity against TPE in yeast cells when fused to the yeast Gal4 DNA binding domain. In the work presented here, we describe a dual-reporter system to assess the boundary activity of proteins such as CTF1 at human telomeres. When located between the two reporter genes, CTF1 shields the telomere distal gene from TPE, while the telomere-proximal gene remains silenced by telomeric heterochromatin. The boundary activity of CTF1 is shown to act regardless its function of transcriptional activator, by opposition to the transcriptional activator VP16 which activates indifferently both transgenes. Moreover, this study shows that CTF1 boundary activity is linked to its H3 binding function, as expected from a chromatin remodeler.

ChIP experiments showed that histone deacetylation is the main histone modification involved in gene silencing at mammalian cell telomeres. Distinctly to yeast cells, the histone deacetylation signal in human cells extented over a short range along the chromosome. CTF1 may help to block this propagation and therefore to restore histones acetylation level on telomere protected *locus*. Surprisingly, other histone marks such as trimethyl-H3K9 or trimethyl-H4K20 were found on telomere protected *locus*, while in another clone, unsilencing of telomere distal transgene was associated with recruitment of the histone variant H2A.Z.

Thus, I conclude that CTF1 displays a chromatin boundary function which is independent of its transcriptional activity and therefore exhibit features required for use as chromatin insulator in biotechnological applications.

RESUME

Les génomes eucaryotes sont compartementalisés en domaines structurels qui peuvent affecter positivement ou négativement l'expression des gènes avoisinants. Ces régions dites d'euchromatine ou d'hétérochromatine sont caractérisées par des modifications post-traductionnelles des histones qui peuvent faciliter ou au contraire inhiber la transcription des gènes qui s'y trouvent. Ainsi, isoler un gène de son environnement chromatinien est problème fréquent lorsqu'il s'agit de contrôler son expression dans le cadre d'applications en biotechnologie ou encore en thérapie génique. Certaines régions de chromatine telles que les télomères sont composées d'hétérochromatine constitutive qui mène au silençage des gènes avoisinants. Cet effet de position télomérique (TPE) est connu dans la levure *S.cerevisiae* comme se propageant par auto-recrutement du complexe de déacétylation d'histone SIR, alors que peu de modifications de chromatine ont pu être associées à ce phénomène dans les cellules de mammifères.

Le facteur de transcription CTF1 a montré des propriétés d'anti-silençage dans les cellules de mammifères, ainsi qu'une activité barrière contre le silençage télomérique dans les cellules de levures lorsqu'il est fusionné au domaine de liaison à l'ADN de la protéine de levure Gal4. Dans le travail présenté ci-après est décrit un système à deux gènes rapporteurs permettant de mesurer l'activité barrière de protéines telles que CTF1 aux télomères humains, et les modifications de chromatine qui y sont associées. Lorsque CTF1 est placé entre les deux gènes rapporteurs, le gène distant du télomère est protégé du silençage qui lui est associé, alors que le gène proche du télomère reste soumis à ce silençage induit par l'hétérochromatine télomérique. L'activité barrière de CTF1 est montrée ici comme agissant indépendamment de son activité transcriptionnelle, par opposition à l'activateur transcriptionnel VP16 qui active indifféremment les deux transgènes. En outre, cette étude appuie l'hypothèse stipulant que CTF1 agisse comme remodeleur chromatinien puisqu'elle démontre que son activité barrière est directement dépendante de son activité de liaison avec l'histone H3.

De plus, des expériences d'immuno-précipitation de la chromatine démontrent que la déacétylation des histones est le majeur phénomène intervenant dans le silençage télomérique. Par opposition à la levure, ce signal de déacétylation ne se propage dans les cellules humaines que sur une courte distance le long du chromosome. CTF1 agit ainsi en bloquant cette propagation et en restaurant le niveau d'acétylation des histones sur le *locus* protégé du télomère. De manière surprenante et inattendue, d'autres modifications d'histones telles que

les H3K9 et H4K20 triméthylées sont aussi observées à ce *locus*, tandis le recrutement du variant H2A.Z peut aussi être suffisant à restaurer l'expression du gène distant du télomère.

En terme de cette analyse, CTF1 exhibe ainsi une fonction de barrière chromatinienne qui exclue une activité transcriptionnelle non désirée - propriété qui est requise dans l'établissement des isolateurs visant à permettre le contrôle d'un transgène dans le cadre d'applications en biotechnologies.

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Abbreviations

a.a	amino acid
ACF	ATP-dependant chromatin assembly and remodeling factor
ALT	alternative lengthening of telomeres
ATM	ataxia telangiectasia mutated
ATP	adenosine triphosphate
ARP	actin related protein
BSA	bovine serum albumine
BFP	blue fluorescent protein
CAF1	chromatin assembly factor 1
CENP-A	centromere protein A
ChIP	chromatin immuno-precipitation
CHO	chinese hamster ovary
CMV	cytomegalo-virus
CTCF	CCCTC binding factor
CTD	carboxy-terminal domain
CTF	CCAAT–box-binding transcription factor
DBD	DNA binding domain
DMEM	Dulbecco's eagle medium
DNA	deoxyribonucleic acid
DNMT	DNA methyl transferase
DSB	double strand breaks
DsRed	*Discosoma sp.* red fluorescent protein
DTT	dithiothreitol
EDTA	ethylene diamine tetra-acetate
EGFP	enhanced green fluorescent protein
FACS	fluorescence associating cell sorting
FISH	fluorescent *in situ* hybridization
FRAP	Fluorescence recovery after photobleaching
H2Abdb	Variant of histone H2A (Barr body deficient)
HAT	histone acetyl transferase
HDAC	histones deacetylase
HML/HMR	heterothallic mating type *locus* left/right

HMT	histone methyl transferase
HeLa	Henrietta Lacks' cell line
Hepes	N-2-hydroxyethyl piperazine-N'-2 ethanesulfonic acid
HMT	histones methyl transferase
HP1	heterochromatin associated protein 1
HS4	DNase1 hypersensitive site 4
LSD1	lysine specific demethylase 1
LTR	long terminal repeat
ISWI	imitation switch
MAT	mating type
MBD	methyl binding domain
MRN	Mre11/Rad50/Nbs1
NAD	nicotinamide adenine dinucleotide
NF1	nuclear factor 1
NHEJ	non-homologous end joining
NURF	nucleosome remodeling factor
PCAF	p300/CBP associated factor
PBS	phosphate buffered saline salt solution
PCR	polymerase chain reaction
POT1	protection of telomere 1
PMSF	phenyl methyl sulfonyl fluoride
PRMT	protein arginine methyl transferase
RAP1	repressor activator protein 1
REST	RE-1 silencing transcription factor
RNA	ribonucleic acid
S/MAR	scaffold/matrix attachement region
SAGA	Spt-Ada-Gcn5 acetyl-transferase
scs/scs'	specialized chromatin sequence
SDS	sodium dodecyl sulfate
SIR	silent information regulator
SNF	sucrose non fermenting
STAR	sub-telomeric anti-silencing region
SV40	simian virus 40
SWI	switching mating type
TERT	telomerase reverse transcriptase

TGF-β	transforming growth factor β
TPE	telomere position effect
TRD	TGF-β responsive domain
Tris	tris-hydroxymethyl-aminomethane
TRF1/2	telomeric repeats binding factor ½
USF1	upstream stimulatory factor 1
VP16	herpes simplex virus protein 16
WRN	Werner's syndrome protein

I. INTRODUCTION

I.1 Chromatin structure and consequences on gene expression.

In eukaryotic organisms, genes are transcribed at various levels. Mechanisms that lead to the control of gene expression depend partly on the chromatin structure and its regulation through complex and diverse pathway. Chromatin structure governs how DNA is packed inside the *nucleus* and is consequently the foreground step that influences the next levels of gene expression regulation.

I.1.1 Chromatin structure in higher eukaryotes.

I.1.1.1. The fundamental nucleosome unit.

In eukaryotic cells, DNA is compacted in a complex chromatin structure whose fundamental unit is the nucleosome. Each nucleosome is wrapped by 1.75 turns of DNA helix that represent 147bp (Luger et al., 1997; Noll, 1977; Shaw et al., 1976). The organization in nucleosome units results in a DNA compaction that can reach up to 10-fold as compared to native DNA (Kornberg, 1974). Then, one face of DNA helical is hidden and sequence recognition is consequently more difficult, but DNA accessibility is however allowed by destabilization of the nucleo-proteic complex either by over-twisting of the DNA helical or by formation of DNA loop that would spread on nucleosome surface (Langst and Becker, 2004). The nucleosome complex is composed of two H2A/H2B heterodimers and one (H3/H4)2 tetramer (Fig.1). Histone H1 stays independent of the histone core and is positioned at the entry and the exit of DNA from histone octamer to seal DNA to the nucleosome particle. Histones proteins have been reported to be highly conserved and only a few variants are described. These variants are generally associated with important functional modifications and concern principally H2A and H3, whereas the lower number of H2B variant and the absence of H4 isoforms is assumed to be imposed by the necessity of interactions between H2B and H4 to associate H2A/H2B and H3/H4 tetramers (Luger et al., 1997).

Among all histones proteins, H2A shows the most diverse variant which display important roles in chromatin organization. Some of H2A variants are disposed within the genome in a very localized manner, particularly MacroH2A whose presence is predominant on the inactive X chromosome. The involvement of MacroH2A in heterochromatinization of inactive X chromosome is not clearly demonstrated but MacroH2A acts probably by making the

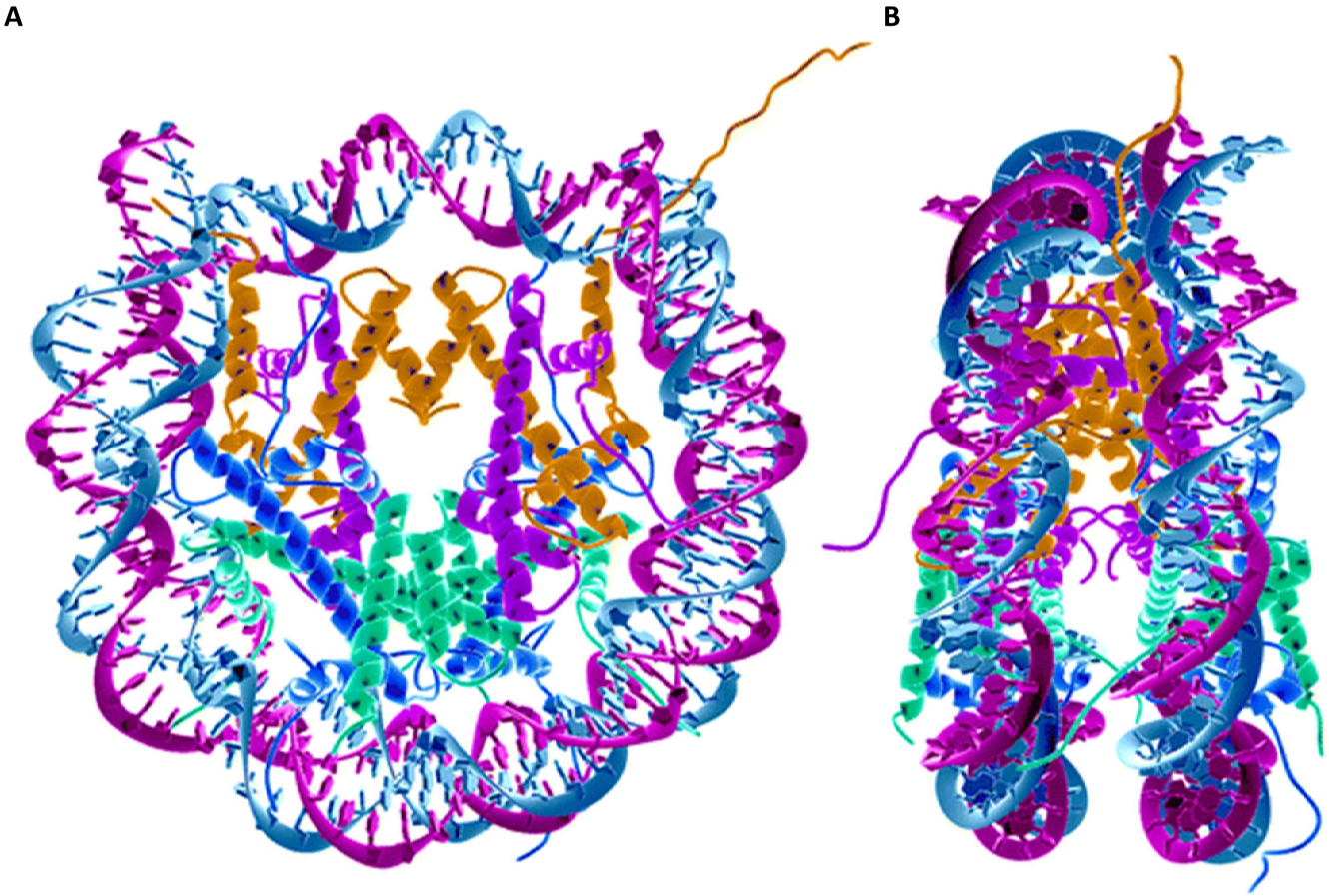


Figure 1. The nucleosome core particle.
Front (A) and side (B) views of the nucleosome core particule. The nucleosome core is composed of two H2A/H3B dimers and one (H3/H4)2 tetramer. DNA (blue/violet double helix) turns around the nucleosome core on 147bp. H2A and H2B histones are respectively represented in night and light blue, while H3 and H4 are respectively represented in yellow and violet. Wrapping of DNA around nucleosomes allows a 10-fold compaction of DNA. (Adapted from Luger *et al.*, 1997).

nucleosome more stable. By opposition to MacroH2A, nucleosomes containing the H2Abdb variant associates predominantly with active X and autosomal chromosomes. Its presence in euchromatin regions is actually explained by shorter wrapping of DNA around H2Abdb that represents only 118bp instead of the current 147bp (Bao et al., 2004), as its shortened C-terminal tail may confer a weaker nucleosome structure. Other H2A variants have widespread chromosomal localization. H2A.X or H2A.Z are conserved in many organisms from yeast to mammals and can represent each up to 10% of total H2A histones. H2A.X is involved in double strand breaks (DSB) repair and is preferentially phosphorylated by the ATM complex present at sites flanking DSB (Rogakou et al., 1999). Even if it does not directly mediate DSB repair, phosphorylated H2A.X may facilitate non-homologous end joining process (NHEJ) by destabilizing local chromatin (Redon et al., 2002). H2A.Z is also an important H2A variant whose deletion leads to genome instability (Carr et al., 1994; Rangasamy et al., 2003). Despite a presence in all the genome, H2A.Z is preferentially distributed at centromeric and pericentromeric chromatin, suggesting an implication in chromosome segregation (Rangasamy et al., 2003; Rangasamy et al., 2004). On the other hand, H2A.Z is also present at intergenic regions, and it is associated with both transcriptional activation and repression (van Daal et al., 1988). Indeed, H2A.Z has firstly been shown to be present at heterochromatin *loci* in *Drosophila* and to interact with HP1α at inactive promoters in mammalian cells (Fan et al., 2004). Secondly, H2A.Z has been shown to block SIR-mediated silencing and to active silent promoters in yeast (Meneghini et al., 2003; Zhang et al., 2005), although it is more correlated with active promoters in human cells (Barski et al., 2007). Then H2A.Z appears as an ambivalent histone variant that might be relevant of chromatin boundaries (Barski et al., 2007; Li et al., 2005).

Among histone H3 variants, CENP-A and H3.3 are the most abundant isoforms in mammals. CENP-A manages a crucial role in centromere formation and it is required for accurate chromosome segregation (Kamakaka and Biggins, 2005). Its depletion in *C.elegans* leads to kinetochore null pheotype where most of kinetochore-associated proteins are mislocalized (Oegema et al., 2001). The variant H3.3 can represent up to 25% of total H3 in *Drosophila* cell line and is deposited onto the DNA independently of the cell cycle and DNA replication, unlike H3.1 and H3.2 variants (McKittrick et al., 2004). The role H3.3 in gene expression is not completely known but it is associated with gene activation in *Drosophila* and it may be essential for gene regulation. Studies have also shown a possible

role of H3.3 in chromatin boundaries establishment by positioning at histones replacement *loci* (Mito et al., 2007).

Variants of H1 histone are relatively rare and generally associated with gene repression. The most known of these variants are H1° and H5, which are respectively sperm and testis specific variants (Wagner et al., 1977). Interestingly, H1 variants may have evolved to a cell type specific chromatin organization, by opposition to the H2A and H3 variants, which are generally expressed in most of cells.

I.1.1.2. Non nucleosomal proteins.

Non-histone proteins act also to organize chromatin structure, particularly the chromodomain-containing proteins and the Sirtuin family members. The chromodomain was initially defined as a 50 amino-acids region of homology in HP1- and Polycomb-likes proteins in *Drosophila*, and it has been extended to SUV39 histone methyl-transferases, CHD, and Retinoblastoma binding protein 1 families (Jones et al., 2000). HP1 family constitutes an important class of structural chromatin proteins whose loss of chromodomain function causes cell death in mammals (Filesi et al., 2002). HP1α and HP1β have been associated with constitutive heterochromatin in mammals whereas HP1γ is excluded from constitutive heterochromatin but distributed as heterochromatin-like complexes within euchromatin regions. Although HP1 proteins are involved in the establishment of heterochromatin formation, their presence does not inhibit directly the accessibility of transcription factors to DNA (Cheutin et al., 2003), but it acts to allow interactions with other proteins, bridging cellular signals to chromatin structure modifications. For instance, HP1α chromodomain interacts with HP1α itself as well as histone H3 methylated on Lys 9 residue (H3K9), stabilizing thus the heterochromatin by linking the nucleosomes between each other (Perrini et al., 2004; Zhao et al., 2000). Piacentini *et al.* (2003) has even described HP1-mediated gene activation on Hsp70 *locus* in *Drosophila*, confirming a multi-functional role of HP1 in chromatin structure (Piacentini et al., 2003).

Sirtuin proteins have been particularly well studied in *S.cerevisiae* in which they form a complex that acts to structure and propagate heterochromatin (Hoppe et al., 2002). This propagation of heterochromatin is facilitated by the histone deacetylase activity of Sir2 (Kristjuhan et al., 2003; Smith et al., 2000; Xu et al., 2007). Even if the SirT1 to SirT7 human homologues share the same catalytic domain as Sir2, they are not yet described as chromatin

components in higher eukaryotes and they display more various activities and localizations. For instance, SirT2 is known to deacetylate tubulin in the cytoplasm whereas SirT3, SirT4 and SirT5 are only located in mitochondria (Michishita et al., 2005). Only the three isoforms SirT1, SirT6 and SirT7 are present in the *nucleus* where they exhibit an important role in chromatin organization through histones deacetylation. SirT1 and SirT6 knockouts exhibit marked phenotypes as SirT6 deficiency leads to genomic instability and premature cellular senescence that may result from its involvement in DNA repair and telomeres integrity (Lombard et al., 2008; Michishita et al., 2008). SirT1 promotes the formation of facultative heterochromatin through histones deacetylation and it deacetylates also other non histone proteins such as tumor suppressor p53 (Vaziri et al., 2001),

I.1.2 Chromatin modifications influencing gene expression.

Nucleosomes compaction is thought to reflect so-called “open” or “closed” states of chromatin. Generally, genes located in an open chromatin region are considered as more accessible to transcription factors and to the basal transcription machinery, whereas those located in a closed chromatin context are less accessible and easily silenced. Nucleosomes compaction can be modified either by a direct ATP-dependent mechanism, such as that of nucleosome remodeling complexes, or by an indirect pathway involving post-translational histones modifications. The latter pathway is more complex because of the numerous modifications leading to the creation or abolition of binding sites for other structural and chromatin modifying proteins, whereas the combinations of modifications and interactions it involves is commonly called the histone code.

I.1.2.1 Chromatin remodeling complexes.

Chromatin remodeling complexes are able to modify chromatin by moving or ejecting nucleosomes, which may facilitate the accessibility of the transcription machinery to the DNA. Other chromatin remodeling complexes are able to remove H2A-H2B dimers from the nucleosome to permit their replacement by dimers containing the H2A variant (Table 1).

Chromatin remodeling complexes share the catalytic subunit of the SF2 ATP-dependent translocases but they lack a helicase activity. The most studied family of chromatin remodeling complexes is the SWI/SNF family that is conserved from yeast to Human. SWI/SNF complexes are known to remodel chromatin by favoring random nucleosome positioning and nucleosome ejection for proper gene activation, although their presence is

Table 1. ATP-dependant remodeling complexes (adapted from Vaquero et al. 2003).

Complex	Organism	Subunits	Function	references
CHRAC	*D.melanogaster* *H.sapiens*	Acf-1, ISWI, CHRAC16,	Shares ACF activities.	(Varga-Weisz et al., 1997)
RSF	*H.sapiens*	Rsf-1 (p325) hSNF2H	Allows formation of competent transcription initiation complexes in vitro. Assembles chromatin in vitro. Binds to H3/H4.	(LeRoy et al., 1998; Loyola et al., 2001)
NCoRC	*H.sapiens*	Tip5 hSNF2H	Induces nucleosome sliding, Tip5 interacts with the RNA Polymerase I terminator factor TTF-I.	(Strohner et al., 2001)
ACF	*D.melanogaster* *H.sapiens*	Acf-1, ISWI (hSNF2H in human)	Assembles chromatin in vitro in the presence of the histone chaperone NAP-1. Slides nucleosomes and activates chromatin transcription.	(Ito et al., 1997; Ito et al., 1999)
SWI/SNF	*S.cerevisiae* *D.melanogaster* *H.sapiens*	Brg1/Brm about 10 subunits	Alters nucleosome structure. Contains actin related SWI2/ proteins.	(Olave et al., 2002)
INO INO80	*S.cerevisiae*	12-polypeptide complex	Chromatin remodeling, facilitates transcription in vitro, contains 3’ to 5’ DNA helicase activity.	(Shen et al., 2000)
CHD1	*S.cerevisiae* *H.sapiens*	CHD1	Interacts with the human FACT subunit SSRP1. Chromatin remodeling activity.	(Kelley et al., 1999; Tran et al., 2000)
NuRD	*D.melanogaster* *H.sapiens*	CHD4 (Mi2) MTA2, MBD3, HDAC1/2, RbAp48/46	Histone deacetylase and chromatin remodeling activity. Interacts with methylated DNA through interaction with MBD2.	(Wade et al., 1999; Zhang et al., 1998)

rarely required to activate gene transcription in yeast (Becker and Horz, 2002). In mouse and Human, only two SWI/SNF complexes are described. The human BAF and PBAF complexes contain 11 subunits, whose respective BRG1 and hBRM ATPases share 75% of homology and whose Actin-Related Proteins (ARPs) suggests a probable link to the nuclear matrix (Olave et al., 2002; Peterson et al., 1998). Nevertheless, the number of subunits in SWI/SNF complexes and the possible interactions with numerous other proteins imply varied roles in gene activation, which may depend on distinct subunits. In mammals, several classes of transcription factors are able to recruit SWI/SNF complexes to the DNA, as exemplified by the MyoD activated genes in fibroblasts (de la Serna et al., 2001a; de la Serna et al., 2001b). Krebs *et al.* (2000) showed that SWI/SNF complex is in turn able to recruit a histone acetyltransferase to the promoter of a large proportion of the genes expressed in the late G2/M phase of the cell cycle (Krebs et al., 2000).

The ISWI family is characterized by a nucleosome positioning action that results in equal DNA distances between nucleosomes. In *Drosophila*, null mutations of ISWI are lethal and the absence of colocalization of ISWI with the RNApolII bulks suggests that the main role of ISWI complexes is not transcriptional regulation (Deuring et al., 2000).

In opposition to SWI/SNF family, NURD complexes establish a repressive chromatin environment, such as the repression of homeotic genes by the polycomb system during *Drosophila* early development (Kehle et al., 1998). NURD activity relies on subunits that are able to reposition nucleosomes and also on other subunits, like HDAC1 or HDAC2, involved in histone deacetylation. Moreover, the role of NURD complexes in heterochromatin establishment and propagation is confirmed by the presence of the MBD2 and MBD3 subunits that interact preferentially with DNA methylated on CpG residues (Zhang et al., 1999). Interestingly, such complexes are unusual in that they lead to histone deacetylation by an active ATP-dependent mechanism.

Finally, other chromatin remodeling complexes are more specialized, like SWR1 and INO80, which are respectively able to replace H2A-H2B dimers by H2AZ-H2B and to reverse this reaction in yeast (Wu et al., 2005). Then, chromatin remodeling complexes appear to be targeted chromatin modifiers whereas nucleosomes are passive, and their range of action is often restricted in time or space, and dependent of an adequate recruitment at specific *loci*.

I.1.2.2 Histone acetylation and deacetylation.

In chromatin organization, histone acetylation represents a major factor of regulation, and histone acetylation is correlated with active chromatin regions, whereas histone hypoacetylation corresponds to a less accessible and consequently more silent chromatin. The reason of increased DNA accessibility when histones are acetylated may be the consequence of the neutralization of positive charges on lysine residues, so that interactions between histones tails and DNA would be reduced, and compaction of chromatin in 30nm fiber avoided (Felsenfeld and McGhee, 1986).

Newly synthesized histones are acetylated on lysine residues at their N-terminal tail, and they are rapidly deacetylated after deposition onto the DNA (Annunziato and Seale, 1983). Lysine residues are acetylated by histone acetyl-transferases (HAT) (Table 2). Six families of HATs have been described in mammals, among which GNAT, TAF250 (a TFIID subunit) and CBP/p300 families contain a bromodomain that interacts with acetylated H4 peptide. Most HATs are incorporated in multi-subunits complexes whose other components permit to modulate their specificity. For example, Gcn5 alone is able to acetyl H3K14 only, while its integration in SAGA or ADA complexes leads to the acetylation of H3K9,14,18,23 and H3K9,14,18, respectively (Grant et al., 1999). In the MYST family, Esa1 and Ip60 acetylate only free H2A, H3 and H4, but they can acetylate nucleosomal H2A and H4 when integrated in their native complexes (Allard et al., 1999; Ikura et al., 2000). The GNAT and MYST families are the most studied in mammalian cells because of their frequent association with transcription factors such as SAGA which is known to be recruited by the c-myc and E2F families of transcription activators (Lang et al., 2001; McMahon et al., 2000). Both GNAT and MYST families have been shown to act also in DNA repair (Brand et al., 2001; Utley et al., 1998).

Acetyl-residues are removed from acetyl-lysines by Histones Deacetylases (HDAC) that belong to four major categories: the histones deacetylases class I, II and IV that are characterized by a zinc-containing catalytic site, and the histones deacetylases class III that belongs to the family of NAD-dependent Sirtuin deacetylases (Table 3). HDAC class I (HDAC1, 2, 3 and 8) are limited to the *nucleus,* where they are needed for cell survival and

Table 2. Histones acetyl-transferases complexes

Complex	Organism	Catalytic subunit	Function	references
PCAF	*H.sapiens*	PCAF (HAT+ BrD)	transcriptional activation	(Carrozza et al., 2003; Kornberg and Lorch, 1999)
STAGA	*H.sapiens*	hGcn5L (HAT+ BrD)	transcriptional activation	(Carrozza et al., 2003; Kornberg and Lorch, 1999)
TFTC	*H.sapiens*	hGcn5L (HAT+ BrD)	transcriptional activation	(Carrozza et al., 2003; Kornberg and Lorch, 1999)
Tip60	*H.sapiens*	Tip60 (HAT + CHD)	transcriptional activation / DNA repair	(Carrozza et al., 2003; Kornberg and Lorch, 1999)
MSL	Mammals /fly	MOF (HAT + CHD)	Dosage compensation	(Carrozza et al., 2003; Kornberg and Lorch, 1999)
p300/CBP	Mammals	p300/CBP	transcriptional activation	(Kimura et al., 2005; Kornberg and Lorch, 1999)
TAFIID	Mammals /fly	TAFII250 (TAF1)	RNA pol II transcription	(Kimura et al., 2005; Kornberg and Lorch, 1999)
TFIIIC	Mammals	hTFIIIC110 hTFIIIC90	RNA pol III transcription	(Kimura et al., 2005; Kornberg and Lorch, 1999)
ATF-2	Mammals	ATF-2	transcriptional activation	(Kimura et al., 2005; Kornberg and Lorch, 1999)

Table 3. Histones deacetylases complexes

Complex	Organism	Catalytic subunit	Function	references
mSin3	*H.sapiens*	HDAC1, HDAC2	Transcriptional repression	(Kornberg and Lorch, 1999; Laherty et al., 1997)
NURD	*H.sapiens*	HDAC1, HDAC2	Transcriptional repression, nucleosome remodeling	(Bowen et al., 2004; Kornberg and Lorch, 1999)
N-CoR/ SMRT	*H.sapiens*	HDAC3	Transcriptional repression	(Jonas and Privalsky, 2004; Kornberg and Lorch, 1999; Tomita et al., 2004)
Hda1-like	*H.sapiens*	HDAC3/4/5	Transcriptional repression	(Kornberg and Lorch, 1999)
SirT1	Mammals	SirT1	Role in mammalian development and differentiation, involved in cell survival through p53 regulation	(Cheng et al., 2003; Luo et al., 2001; Vaziri et al., 2001)
SirT6	Mammals	SirT6	Maintenance of telomeric heterochromatin, involved in DNA repair	(Lombard et al., 2008; Michishita et al., 2008)

proliferation. For example, HDAC1 and HDAC3 are known to enhance hypoxia inducible factor 1α *via* direct interaction with this transcription factor, and HDAC2 is involved in the modulation of transcription by regulating p53 binding activity (Dokmanovic et al., 2007). Histones deacetylases class II (HDAC4,5,6,7 and 10) shuttle between *nucleus* and cytoplasm, and they display a cell type specificity, as illustrated by HDAC4 which is involved in chondrocyte differentiation in mouse (Vega et al., 2004) and HDAC7 whose knockout causes defects in vascular integrity (Chang et al., 2006). The Sirtuin class of histones deacetylases is characteristized by their NAD-dependent activity. In mammals, only SirT1, SirT6 and SirT7 are located in the *nucleus* (Michishita et al., 2005). SirT1 is considered as the main nuclear NAD dependent deacetylase and it targets mainly H1K26, H3K9 and H4K16 in human cells (Michishita et al., 2005; Vaquero et al., 2004) whereas SirT6 is located in heterochromatin regions, facilitating heterochromatin formation by mediating H3 deacetylation. Moreover, SirT1 show a preferential affinity for acetylated H4K16 which is crucial for heterochromatin establishment and it regulates Suv39h1 to mediate H3K9 methylation (Vaquero et al., 2007; Vaquero et al., 2004).

I.1.2.3 Histone methylation.

Histones are frequently methylated on lysine and arginine residues, so that lysine residues can be either acetylated or methylated. Moreover, lysine residues can be mono-, di- or tri-methylated, while arginines can be di-methylated in a symmetric or asymmetric configuration, which increases the complexity of the histone code. Unlike acetylation, methylation does not affect lysine charges and it may thus have distinct consequences with regard to the chromatin folding. This likely explain why histone methylation cannot be associated to transcriptional active or silent chromatin, and consequently that each methylation rather serves to mark histones to address further modifications to the local chromatin.

Lysine methylation is carried out by the histones methyltransferases (HMT) that act to modify specific target residues, and which are classified accordingly (Table 4). For instance, Suv39h1/2 and G9a catalyze respectively tri- and di-methylation of H3K9 while Dot1 methylates only H3K79 (Ng et al., 2002; Rice et al., 2003; Tachibana et al., 2005). On the other hand, arginine methylases are distinguished in type I or II depending on whether they methylate arginines with an asymmetrical or symmetrical configuration, and they target distinct substrates as other HMT, although their catalytic mechanism and specificity remain partially unknown. For instance, methyl transferase PRMT1 (Protein arginine Methyl-

Table 4. Histones methyl-transferases

Enzyme	Organism	Substrate	Function	references
CARM1	*H.sapiens*	H3R2 (*in vitro*)	Transcriptional activation	(Chen et al., 1999; Schurter et al., 2001)
	M.musculus	H3R17		
SET7/	*H.sapiens*	H3K4	Transcriptional activation	(Nishioka et al., 2002; Wang et al., 2001a)
Set9				
Suv39h1/2	*M.musculus*	H3K9	Pericentric heterochromatin	(Lachner et al., 2001; Peters et al., 2001)
G9a	*M.musculus*	H3K9	Imprinting, transcriptional repression	(Tachibana et al., 2001; Tachibana et al., 2002)
		H3K27		
Dot1/	*H.sapiens*	H3K79	Telomeric silencing, pachytene checkpoint	(Lacoste et al., 2002; van Leeuwen et al., 2002)
DOT1L	*S.cerevisiae*			
PRMT1	*H.sapiens*	H4R3	Transcriptional activation	(Strahl et al., 2001; Wang et al., 2001b)
Pr-SET7/	*H.sapiens*	H4K20	Transcriptional silencing mitotic condensation	(Fang et al., 2002; Nishioka et al., 2002)
Set8	*D.melanogaster*			
Ash1	*D.melanogaster*	H4K20	Trithorax activation (in concert with H3-K4 and H3-K9 methylation)	(Beisel et al., 2002)

transferase) may recognize repeats of Arg-Gly-Gly sequences, and they may be able to methylate some but not all arginine residues (Najbauer et al., 1993).

Only few histone demethylases have been identified so far and histone methylation has been considered as a long term or permanent chromatin modification, partly because of its presence at heterochromatin of the inactive X chromosome, or at the centromeric regions. Histones demethylases are less residue-specific than their respective methylases. For instance, the transcriptional repressor REST links SMCX that demethylates H3K4Me3 to genes implied in X-linked mental retardation (Tahiliani et al., 2007), but Lysine Demethylase 1 (LSD1) is able to demethylate mono and di-methyl H3K4, and to lesser extent, mono- and di-methyl H3K9. Thus, LSD1 homologs have been associated with both active and repressed transcription in yeast (Chosed and Dent, 2007). The dual role of LSD1 in gene transcription is moreover confirmed by its regulation of heterochromatin-euchromatin boundaries (Chosed and Dent, 2007; Lan et al., 2007). Similarly, JMJD2A demethylates both H3K9Me3 and H3K36Me3, although its overexpression has been shown to abrogate HP1 recruitment, which rather suggests a role in transcription activation (Klose et al., 2006).

I.1.2.4 DNA methylation.

DNA methylation occurs in many organisms, and it occurs only on cytosine of CpG dinucleotides in mammals. How DNA methylation is associated with long term gene silencing is not fully understood but such silencing effect may be necessary for chromatin organization. Indeed, many *loci* are known to be methylated like transposable elements, repeated DNA sequences including centromeric region, inactive X chromosome or even gene-containing regions (Bird, 2002; Goll and Bestor, 2005; Rabinowicz et al., 2003).

DNA is methylated by DNA methyl-transferases (DNMT) isoforms comprising Dnmt1, Dnmt2 and Dnmt3. Some like Dnmt3a and Dnmt3b are only able to methylate *de novo* DNA, except in mouse embryo in which they also act to maintain methylation (Chen et al., 2003). Dnmt1 is able to maintain DNA methylation and to carry *de novo* methylation, while Dnmt2 is less characterized and knockouts mice for this enzyme do not show noticeable effects on DNA methylation (Okano et al., 1998).

Several proteins interact with methylated DNA or with the DNA methyl-transferases to mediate further modifications that lead to the establishment of heterochromatin. Indeed, proteins containing methyl-CpG binding domain (MBD) interact directly with methylated

DNA (Bird, 2002). Suv39h1/2 methyl-transferases are known to interact with Dnmt3a and Dnmt3b in mouse (Fuks et al., 2003; Lehnertz et al., 2003) and G9a methyl-transferase associate with Dnmt1 insuring a direct positive feedback of H3K9 methylation and DNA methylation on each other (Esteve et al., 2006). Accordingly, studies in Neurospora suggest that DNA methylation may occur as a consequence of H3K9 methylation (Tamaru and Selker, 2001).

I.1.2.5 Other modifications.

Other modifications of chromatin components have been described, like histone ubiquitination or phosphorylation. These modifications are less frequent, although they display also crucial roles in chromatin organization. For instance, phosphorylation on H3S10 is involved in both gene transcription at c-fos and c-jun *loci* and cell division, even if it requires opposite effects on chromatin compaction (Mahadevan et al., 1991; Schmitt et al., 2002). Histone phosphorylation has also been well described in the context of DNA double strand breaks in mouse, in which H2A.X is maximally phosphorylated on Ser139 within 10 min during the DSB repair by NHEJ process (Rogakou et al., 1998).

Ubiquitin is a 76 amino acids protein involved in proteasome recognition and degradation. Histones affected by ubiquitination are mainly H2A and H2B, and effects leading to both transcription activation and repression have been observed. How ubiquitin is involved in transcriptional regulation remains partially unknown but its opposing effects suggest it acts to induce other histones modifications or to recruit other proteins rather than to mediate directly chromatin folding (Zhang, 2003). Histone ubiquitination effects are considered to depend on the genomic position.

I.1.3 Breaking the histone code.

Histone acetylation concerns only a few lysine residues and it is largely associated with a permissive chromatin environment, but other histone modifications are more diverse, and most of their effects on gene expression are not fully understood. Although recent techniques such as ChIP-sequencing have enlightened the general localization of several histones marks within the human genome, particularly concerning histone methylation, interactions of such histones marks with the various chromatin modifiers remain mostly unclear.

Firstly, single histones marks have been associated with preferential genomic functions. For instance, gene expression has been correlated with presence of trimethylated H3K36, H4K20Me1 and methylated H3K4, but with different localization with regard to the transcription start site (TSS) (Fig.2). Indeed, H3K36Me3 and H3K20Me1 are present a few hundreds of base pairs downstream of the TSS of active genes, and it further spreads for several thousands of base pairs on the coding sequence, suggesting that it might help transcription elongation, although H4K20Me1 is more widespread than H3K36Me3 (Barski et al., 2007). Moreover, methylated H3K4 localizes closer to the TSS, and this varies depending of the number of methyl residues. For instance, H3K4Me3 is most abundant in a range of ~300p around the TSS of active genes, while H3K4Me2 and H3K4Me1 are bordering each side of H3K4Me3 localization, suggesting that methylated H3K4 may be directly involved in RNApolII recruitment. Histones marks present in heterochromatin regions are mainly di- and tri-methylated H3K9 and H3K27 since their presence is more frequent in lowly expressed coding regions (Barski et al., 2007). Interestingly, histones marks H3K4Me3 and H3K27Me3 are colocalized in mouse embryonic stem cells while their differentiated embryonic fibroblast and neural progenitor cells counterparts show either H3K4Me3 or H3K27Me3 histones marks on expressed or silenced genes, respectively (Mikkelsen et al., 2007). Moreover, mouse embryonic fibroblasts display also the presence of H3K36Me3 on the transcribed region, as well as H3K4Me3 at the TSS.

Nevertheless, the studies of isolated histones marks does not allow full understanding of the numerous interactions that occur between them and with chromatin modifying protein, yielding more complex combinations of chromatin patterns. For instance, HP1 recognizes specifically H3K9Me3 *via* its methyl-lysine binding domain, but it localization does not always correlate with H3K9Me3 (Cowell et al., 2002; Perrini et al., 2004). This does not exclude an active maintenance of heterochromatin structure by HP1 as observed in FRAP assays in *Drosophila* (Cheutin et al., 2003), while being consistent with observations showing that HP1 binding may be regulated by many distinct mechanisms, like HDAC inhibition that decreases the levels of some HP1 isoforms and increases in turns thoses of dimethylated H3K4 and acetylated H3K9 in human cells (Bartova et al., 2005). Moreover, numerous other proteins are also able to bind methylated lysines such as Tudor- and MBT domains-containing regulators (Kim et al., 2006), yielding quite diverse chromatin modifying pathways making from just one histone modification.

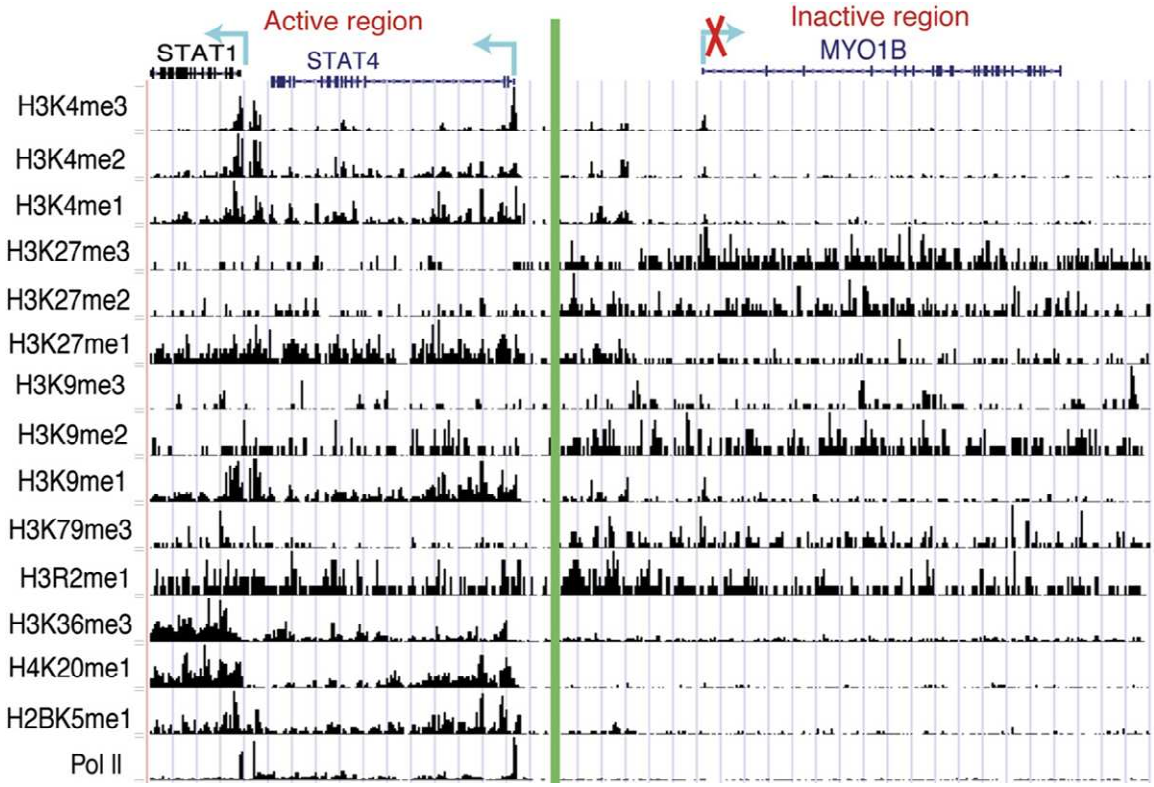


Figure 2. Involvement of main histone marks with regard to gene expression.
ChIP sequencing data representing the occurrence of RNApolII or other histone marks on genes that are highly transcribed (left, STAT1 and STAT4) or not expressed (right, MYO1B). Trimethylated H3K4 is correlated with RNApolII location, while di- and mono-methylated H3K4 are less specific of the transcription start site. In opposition, trimethylated H3K36 and monomethylated H4K20 are absent before transcription initiation and increase just after TSS, although H4K20Me1 level is not maintained over a long distance after transcription initiation when compared to H3K36Me3. H3K9 and H3K27 are more present on the active part of chromatin when mono-methylated, whereas their di- and tri-methylated forms are more present on the heterochromatin side. (adapted from Barski *et al* (2007).

I.2 Particular chromosomal structures and associated chromatin environments.

I.2.1 Structure of the centromere.

The centromere is the structure responsible of chromatid cohesion and their separation during mitosis. Before mitosis, sister chromatids are attached by cohesins and their separation is allowed by the kinetochore function that binds the centromere to microtubules. The DNA sequence of centromeres is composed of large and highly repetitive DNA sequences and heterochromatin formation at centromere is required for proper chromatid separation (Allshire et al., 1995; Kellum and Alberts, 1995; Peters et al., 2001).

Chromatin structure at the centromeres vicinity involves several histone variants that are not localized uniformly, and of which H2A.Z plays a central role (Greaves et al., 2007) (Fig.3). In mouse, centromeric chromatin is composed of blocks of H2A.Z/H3K4Me2-containing nucleosomes that extend up to 20Kb, alternating with blocks of H2A/CENP-A-containing nucleosomes that package up to 40Kb of DNA (Greaves et al., 2007). *In vitro*, H2A.Z has been shown to generate more condensed nucleosomal arrays as compared to H2A, and such compaction is considerably strengthened *in vivo* by the binding of HP1α to H2A.Z-containing nucleosomes (Greaves et al., 2006). Thus, the role of H2A.Z-mediated chromatin compaction for proper centromere function seems quite important, since H2A.Z depletion leads also to the loss of HP1α binding and to defects in chromosomes segregation (Rangasamy et al., 2004). Nevertheless, the organization of CENP-A and other histones marks can vary in a chromosome dependent manner, leading to various centromere structures. Such differences are observed on chromosomes that are poor in pericentric chromatin, which may affect the composition of centromeric chromatin (Greaves et al., 2007).

In contrast, CENP-A-containing chromosomes are not present at pericentric chromatin, and blocks of H2A.Z/H3K9Me3 nucleosomes alternate with blocks of H2A/H3K9Me3. Pericentric regions have been frequently associated with heterochromatin assembly. Indeed, H3K9Me3 marks are largely associated with heterochromatin, and H3K9 deacetylation as well as DNA methylation is required for proper H3K9 methylation in pericentric regions. Moreover, the DNA methyl-transferase DNMT1- and HDAC-containing complexes have been shown to be recruited to pericentric region and to be necessary for H3K9 deacetylation

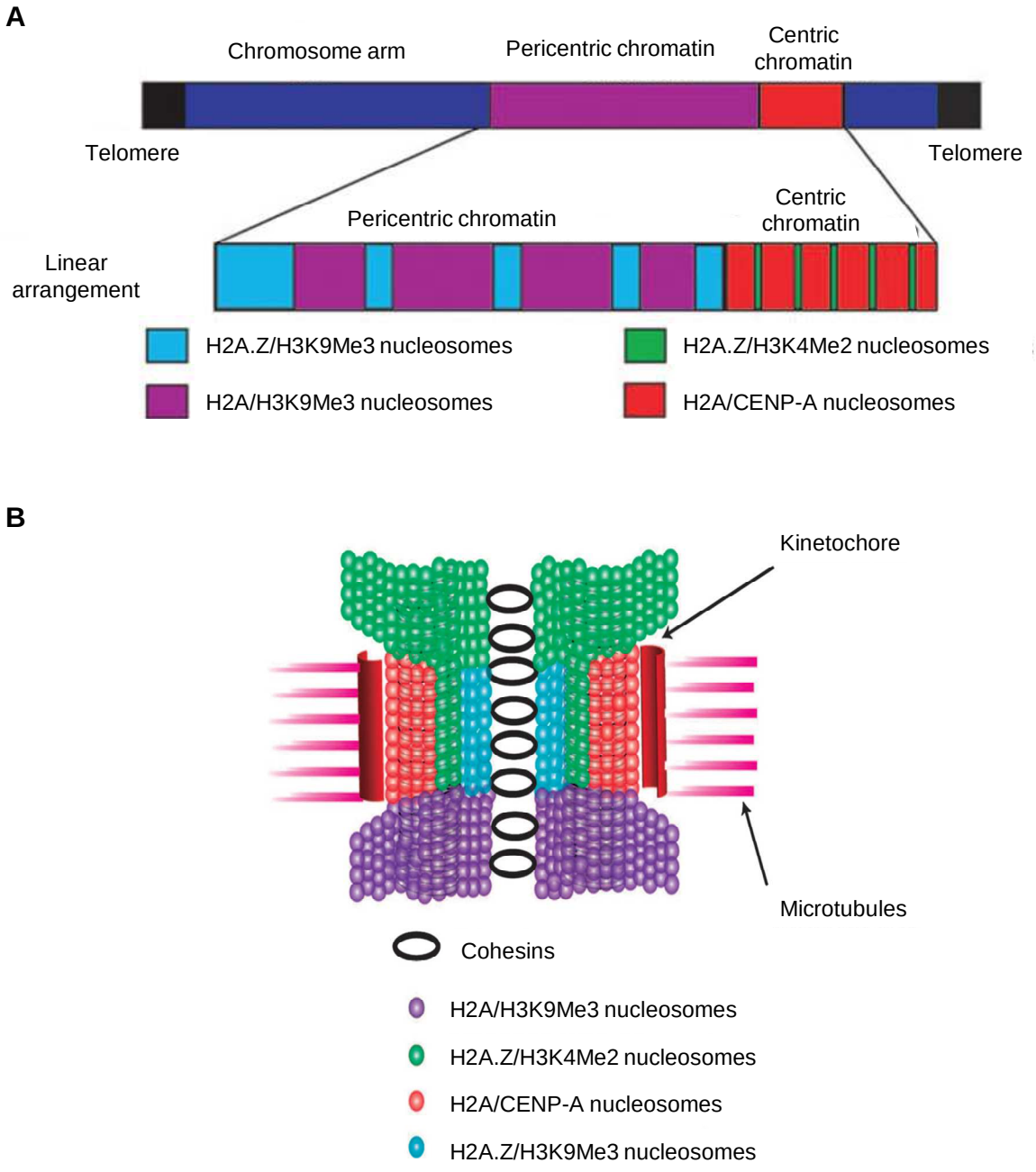


Figure 3. Structure of the centromere and associated heterochromatin.
(A) Schematic representation of the discontinuous linear distribution of H2A.Z at centromeric and pericentric regions. Centromeric chromatin is composed of clusters of H2A.Z/H3K4Me2 nucleosomes alternated clusters H2A/CENP-A nucleosomes, while pericentric chromatin alternates H2A.Z/H3K9Me3 nucleosomes clusters with H2A/H3K9Me3 clusters. (B) Model for the folding of centromeric chromatin in the organization of human inactive X centromere. CENP-A histone variant allows the special function of centromere by linking to the kinetochore. Spatial H2A.Z position is crucial for maintaining the particular 3D organization of the centromere and for proper chromatids cohesion. (Adapted from Greaves *et al.*, 2007).

and methylation (Xin et al., 2004). Nevertheless, other modifications are involved in heterochromatin formation at pericentric heterochromatin in addition to H3K9, since heterochromatin also contains HP1 (Xin et al., 2004). Furthermore, and DNMT3a and DNMT3b are also recruited throughout the cell cycle by their proline-tryptophan-tryptophan-proline motif leading to the methylation of major satellite repeats, suggesting that DNA methylation plays also an important role in heterochromatin establishment in pericentric regions (Chen et al., 2004; Xin et al., 2004). Interestingly, demethylation of H3K9Me3 by the JMJD2b demethylase has been described at pericentric chromatin, where it could participate to the position-effect variegation frequently observed for the expression of genes located at pericentric regions (Fodor et al., 2006).

I.2.2 The inactive X chromosome.

In many organisms including Human, female cells contain two X chromosomes instead of one in male cells and need consequently a dosage compensation by inactivating one of X chromosomes. Dosage compensation involves X chromosome counting and consequently that one X chromosome remains active while the other is mostly converted to heterochromatin. X inactivation is regulated in *cis* by the X inactivation center (Xic) that contains two RNA encoding genes Xist and Tsix that are transcribed in an opposite direction (Lee et al., 1999; Morey et al., 2001) (Fig.4). Studies in human and mouse cells have shown that deleting a X chromosome from a 65Kb deletion downstream of Xist gene leads to its permanent inactivation, showing that Xist gene expression is responsible of the X inactivation. In opposition, Tsix expression would promote its activation. How just one chromosome is activated is attributed to CTCF binding sites located 3' to the Xist gene that would block the effects of an enhancer/silencer located downstream, leaving Tsix expressed (Chao et al., 2002; Percec and Bartolomei, 2002).

The epigenetic status of the inactive X chromosome is not completely identified, but it is strongly correlated with the deposition of MacroH2A histone variant (Costanzi and Pehrson, 1998). The role of MacroH2A in the establishment of heterochromatin likely results from its non-histone macro-domain. Indeed, MacroH2A-mediated X inactivation has been shown to be dependent on Xist RNA expression, suggesting that MacroH2A macro-domain might have a role in RNA-mediated silencing (Csankovszki et al., 1999). In addition, the inactive X is poor in histone modification associated with euchromatin such as H3K4 methylation and H3K9 acetylation, whilst it is enriched in hypoacetylated H4 and other histones marks as

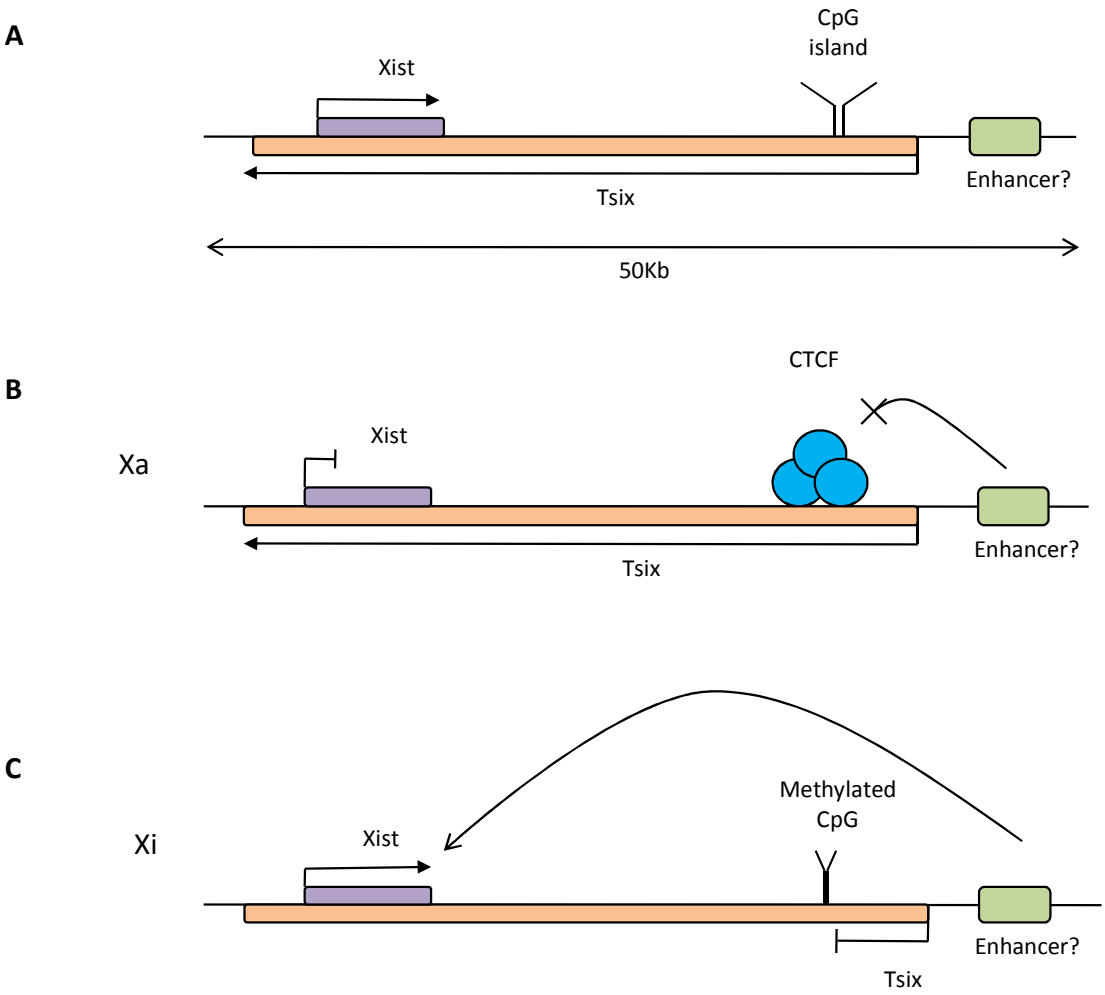


Figure 4. Model of inactivation of chromosome X in mammalian cells.
In mammals, additional X chromosomes of female genomes are counted and heterochromatinized through a mechanism initiated at the X inactivation center (Xic). (A) Xic is a *locus* of 50Kb composed of two RNA-encoding genes disposed in an opposite orientation (Xist and Tsix). CpG islands and close CTCF binding sites are located at the beginning of Tsix gene. (B) Current model displaying how one chromosome X is activated (Xa) whereby CTCF blocks the activation of Xist but not Tsix by a still unidentified enhancer located upstream of Tsix. Binding of CTCF avoids also the methylation of CpG islands, facilitating the expression of Tsix. (C) Alternatively, the inactivated X (Xi) is not bound by CTCF so that Xist is activated by the enhancer and methylated CpG islands inhibit transcription of Tsix.

H3K27Me3, H3K9Me2 and H4K20Me3, all associated with heterochromatin (Heard and Disteche, 2006).

I.2.3 The telomere.

In eukaryotic organisms, linear chromosomes have to be protected against end to end fusion or degradation. This function is insured by the telosome, composed of telomeric DNA and associated proteins.

I.2.3.1 Structure and elongation of telomeres.

In vertebrates, the telomeric DNA is composed of (TTAGGG) sequences repeated for 2-100Kb, but only up to 20Kb in normal human cells (LeBel and Wellinger, 2005). These repeats are preceded by a subtelomeric sequence that may be also involved in telomere-associated proteins recruitment. In many species such as Human or Trypanosome, telomere stability is insured by the formation of T-loop and D-loop structures that hide telomeric repeats and DNA end (de Lange, 2004; de Lange, 2005; LeBel and Wellinger, 2005). In Human, this two-loops structure is maintained by the Shelterin nucleo-proteic complex that binds to other telomere-associated proteins like the WRN helicase and the MRN complex (Fig.5). TRF1 and TRF2 bind to double stranded DNA through a Myb-like domain, POT1 binds only to single stranded DNA, while the other members of Shelterin (RAP1, TIN2 and TPP1) stabilize the complex to seal the telosome structure. Interactions between TRF1 and POT1 (Loayza and De Lange, 2003), as well as interactions between TRF2 and other telomere-associated proteins such as RAP1 (O'Connor et al., 2004), have been associated with the modulation of telomeres extension. Thus, both TRF1 and TRF2 may participate in a stop-elongation signal that could prevent telomeres from increasing their length above an ideal length.

The number of the T_2AG_3 repeats is variable according to the cell type species. Maintenance of the length of telomeric region involves at least two mechanisms. The most prevalent one involves the telomeric reverse transcriptase (TERT) that elongates telomeric DNA using a sequence of complementary RNA as template. This mechanism insures a stable telomeric size and confers a high stability to chromosomal ends. For example, many cancerous cell lines overexpress TERT and studies have shown that the expression of telomerase before apoptosis is sufficient to sort cells out of crisis (Halvorsen et al., 1999). The second mechanism that maintains telomeres length is based on a

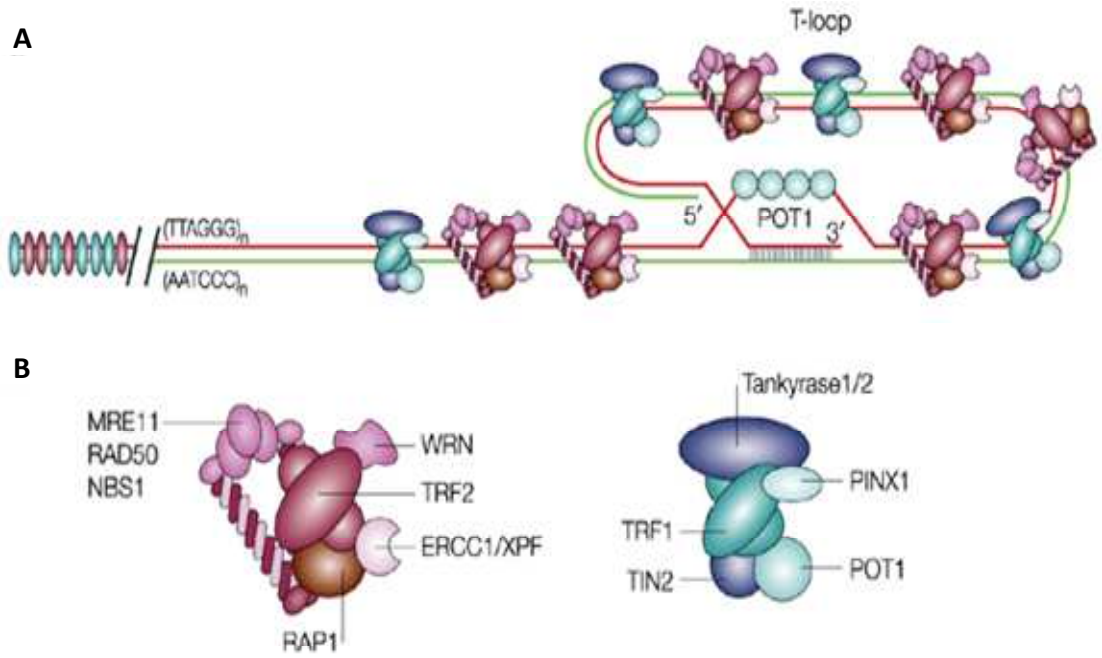


Figure 5. Structure of the nucleo-proteic complex of mammalian telosome.
(A) Structure of human telomeres. Chromosome ends are composed of 2-20Kb of TTAGGG repeats and hidden in a two-loops structure. The T-loop and D-loop (displacement loop) are maintained by the Shelterin complex. Double stranded repeats are bound by TRF1 and TRF2, while single stranded repeats composing the D-loop are protected by POT1. (B) Shelterin includes TRF1, TRF2 and other proteins especially recruited at the telomere such as RAP1 or POT1. The TRF2 containing complex (left) is involved in the protection of chromosome ends, while the TRF1 containing one (right) acts in regulation of telomere length. Interactions between TRF1 and TRF2 containing complexes would be responsible of the maintaining of the two loops structure, but other configurations remain possible. (Adapted from De Lange, 2004)

recombination process that can be different according to the organism studied. For example, in *K.lactis*, the 3' end is elongated using an extrachromosomal circular DNA template, while telomerase independent telomeres extension in dipterian insects is insured by retrotransposition. In Human, such mechanism is not perfectly understood but it probably uses a rolling circle replication and/or it may involve a replication using another telomere as template. This mechanism would be activated by a "crisis signal" elicited by a shorter length of telomeres (Liu et al., 2004; Loayza and De Lange, 2003). Consequently, the cells using only this pathway instead of telomerase based pathway to maintain their telomeres have frequently very short telomeres, and their genome is thus more exposed to genetic aberrations. In addition, studies have shown that telomerase activity inhibits the recombination pathway, rendering the latter pathway as a stand-by mechanism (Ford et al., 2001). Recently, Azzalin *et al.* (2007) has shown that RNA transcribed from telomeric repeats may be also involved in maintenance of telomere integrity (Azzalin et al., 2007), although this mechanism is not completely understood.

I.2.3.2 Telomeric position effect.

The telomeric position effect has been well studied in *S.cerevisiae*, where genes inserted near a telomere are silenced. This silencing is also dependent on the length of the telomere and on its distance to the gene (Kyrion et al., 1993; Renauld et al., 1993). At yeast telomeres and also at other localizations such as at HMR *loci*, the SIR protein complex is a major constituent of heterochromatin and it is responsible for the spread of the telomere-associated silencing. Firstly, Sir4 binds yeast telomeres through its interaction with Rap1, and it then recruits Sir3 and Sir2. Sir2 is known to deacetylate proximal nucleosomes, particularly on H4K16, whose deacetylation is crucial for the formation of heterochromatin, and also H3K56 which is located at the entry-exit point of the nucleosome and may be also required for the establishment of nucleosomal compaction (Xu et al., 2007). This proximal deacetylation results in the binding of these new SIR complexes, and consequently in the spread of further heterochromatic and hypoacetylated structures, depending on the availability of the Sir proteins.

In vertebrates, telomeric gene silencing was first observed in HeLa cells by Baur *et al.* (2001). Despite a controversial study showing that stably expressed transgenes are preferentially inserted at telomeres in CHO cells (Kim and Lee, 1999), the recent observation of TPE in mouse and chicken cells implies that it may occur in most higher eukaryotes, and it

further suggests that the highly expressed transgenes were probably inserted at subtelomeric regions instead of a telomere environment. In human cells, the role of the telomeric length in silencing has also been confirmed, and other studies have shown that this silencing effect is reversible, like in yeast (Baur et al., 2004; Baur et al., 2001). Nevertheless, the distance up to which telomeres may exhibit a TPE remains unknown in mammalian cells. Unlike yeast where the telomere proximity is associated with stronger silencing, no mechanism allowing the spread of a heterochromatin complex from telomere has been observed in mammalian cells as yet.

In any case, the presence of heterochromatin-associated modifications at mammalian telomeres suggests the possibility of a similar spread of heterochromatin from chromosomal ends. Indeed, several studies have shown that the specific histones modifications H3K9Me3 and H4K20Me3 are present at mammalian telomeres, and that H3K9Me3 associates with HP1 to form a heterochromatic complex (Benetti et al., 2007b; Cowell et al., 2002; Perrini et al., 2004; Schotta et al., 2004). On the other hand, HP1 has also been reported to play a role in telomere capping, to prevent telomeres fusion in *Drosophila* (Fanti et al., 1998; Perrini et al., 2004), and to interact with Ku70 that associates in turn with TRF2 at human telomeres (Song et al., 2001). Thus, considering that the H3K9Me3 constitutes also a binding site to the HMT enzymes and also to HP1, a spread of this telomeric heterochromatin in mammalian cells may occur as in yeast. Alternatively, heterochromatin features present at telomeres could simply insure chromosome end protection by the establishment of a compact chromatin structure, since telomerase deficiency, which results in shortened telomeres, is also associated with decreased levels of H3K9Me3 and H4K20Me3 at mouse telomeres (Benetti et al., 2007a).

In mammals, none of the Sir proteins has been localized at telomeres (Michishita et al., 2005), but evidence has provided that several Sirtuin proteins are involved in telomeric heterochromatin formation and in telomere integrity. Indeed, SirT1 has been firstly shown as facilitating the formation of heterochromatin by histone deacetylation and regulation of histone methylation (Vaquero et al., 2007; Vaquero et al., 2004), and SirT6 defect leads to severe diseases that remember those of the deletion of telomere-associated proteins such as WRN - whose lack leads to shorter telomeres and to the Werner aging syndrome (Lieber and Karanjawala, 2004; Machwe et al., 2004). Moreover, SirT6 has recently been shown to deacteylate H3K9 at human telomeres, confirming its involvement in telomeric heterochromatin formation (Michishita et al., 2008).

I.3 Chromatin domain delimiters.

I.3.1 Scaffold/Matrix attachment regions.

The Scaffold/Matrix attachment regions (S/MARs) were initially described as sequences binding to the nuclear matrix or to matrix-associated proteins, dividing the genome in distinct structural domains by the formation of 50-200Kb structural loops. S/MARs are composed of AT rich sequences and their length can vary between 300pb and 3000 Kb. They are exclusively present in higher eukaryotes (Allen et al., 2000; Bode et al., 1995) and could represent up to 100.000 sequences in mammals (Bode et al., 2000). Although MARs are underrepresented inside genes (Rudd et al., 2004), they may be involved in chromatin compartmentalization (Boulikas, 1995) since they may result in the permanent attachment of the chromatin to a solid substrate, the nuclear matrix (Capelson and Corces, 2004).

The presence of MARs in the vicinity of a gene induces an important increase of its expression. For instance, in CHO cells, MARs have been shown to increase both the level of expression of a transgene and the number of cells expressing it, as opposed to silent cells (Girod et al., 2007). According to Bode *et al.* (1995), these regions are different from enhancers on the basis of their lack of activity in transient expression. The model that implies the action of MARs by the formation of distinct structural domains could also account for a possible negative regulation by MARs (Schubeler et al., 1996).

How MARs increase gene expression is not fully understood and their role *in vivo* is difficult to establish because of the lack of a consensus sequence. Nevertheless, their richness in AT sequences makes a region of weaker DNA structure that could facilitate transcription initiation and DNA helix disruption. Such AT sequences can also interact with the Swi1 subunit of the SWI/SNF complex that contains an AT rich interaction domain (ARID) allowing nucleosome remodeling at MARs *loci* (Kortschak et al., 2000). On the other hand, several studies have reported the presence of MAR binding proteins at the nuclear periphery, like MFP1 (Gindullis and Meier, 1999), SAF-A (Lobov et al., 2000), or SAT-B1 (Liu et al., 1997) confirming their proposed role to anchor DNA to the nuclear matrix. Other proteins interacting with MARs could also suggest a possible role in chromatin remodeling. Indeed, MARs have been shown to interact with proteins having a high impact on chromatin organization like and topoisomerase II, but also with high mobility group protein-I/Y or MATH20 that slides along AT repeats to displace adjacent proteins that compact the

chromatin, leaving a more accessible DNA (Hart and Laemmli, 1998). However, whether MARs may delimit distinct chromatin domains remains unclear.

I.3.2 Insulators.

Transcriptional activators are able to activate a gene over long range extent, resulting in a possible activation of other neighboring genes. Although this may lead to position effect variegation, enhancer/silencer blocking elements have been described in most of eukaryotic organisms. How these insulators are able to separate distinct chromatin environment is not clear but two models have been discussed. In the passive model, insulators act only as regulatory domain delimiters, insulating a gene from the effects of neighboring enhancers or silencers (Fig.6A). In this model, insulators may have only a physical role, acting as a constraint or a topological barrier to the activation mechanism of enhancers. In the active model, insulators recruit chromatin modifying proteins like HAT to modify locally the chromatin environment. In this model, the role of insulators is to direct modifications of the chromatin state and to prevent signal propagation (Fourel et al., 2004). These two models are consistent with current studies and are not mutually exclusive. The effect of insulators is crucial for proper gene expression control in the *nucleus*, and many insulators have been described, particularly at *Drosophila* scs and scs' *loci* that bind Zw5 and BEAF insulators proteins (Gaszner et al., 1999; Zhao et al., 1995). Interestingly, some insulators can be modulated, such as the Su(Hw) insulator in *Drosophila* whose efficiency is increased by linking with the Mod protein that inhibits the function of enhancers located on both sides of the Su(Hw) binding site (Gerasimova et al., 1995).

Despite the fact that insulators are generally independent of their orientation, some of them show a polarity in enhancer blocking. For instance, the murine Igf2/H19 insulator has been shown to function more efficiently in its original orientation than when reversed (Hark et al., 2000). On the other hand, between the *Drosophila* iab7 and iab8 *cis*-regulatory domains of parasegmental identity, the combined insulator-enhancer element Fab8 blocks its own enhancer effect on the proximal gene, whereas it leads to increased expression when reversed (Barges et al., 2000). In mouse, the insulator located between Hoxd12 and Hoxd13 show surprising polar features (Kmita et al., 2000). Indeed, in its original orientation, it blocks effects from the downstream Hernia enhancer, but not that of the upstream Digit enhancer. In opposition, when reversed, this insulator blocks Digit effects but not those of Hernia.

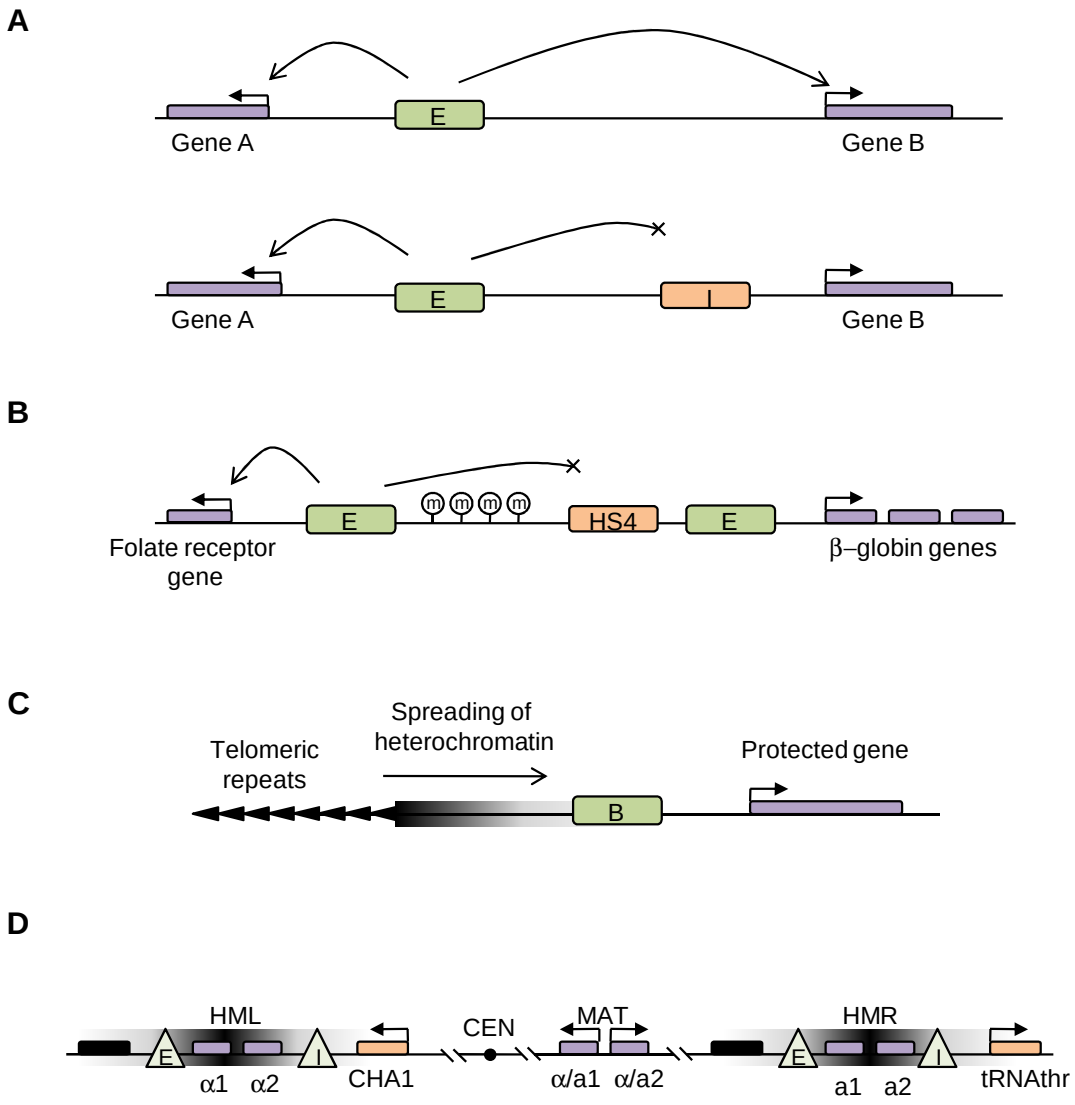


Figure 6. Genome partitioning by insulators and boundary elements.
(A) Insulators are considered as enhancer blocking elements that protect a gene from activation (resp. inactivation) by distal enhancer (resp. silencer). (B) At the chicken β-globin *locus*, β-globin genes are insulated from the enhancer and from the CpG islands present upstream the folate receptor gene by the HS4 insulator. (C) Boundary elements are described as blocking the spread of heterochromatin, for instance from telomeric regions, which protect distal genes from silencing. (D) In *S.cerevisiae*, HML and HMR *loci* are maintained silenced through SIR recruitment by the E and I silencers (respective triangles). Silencing spreading is constrained by $tRNA^{thr}$ and CHA1 genes acting here as boundaries, and leaving adjacent regions such as MAT *locus* in a permissive chromatin state.

I.3.3 Chromatin boundaries.

Boundaries are defined as partitioning the genome in distinct functional domains by blocking the spread of distal heterochromatin (Fig.6C). Like insulators, boundaries are frequently discovered for their capacity to insulate a gene from its chromatin environment, and a gene flanked with boundary elements may be consequently protected from position effects and long term silencing (Bell et al., 2001).

For instance, in haploid *S.cerevisiae*, mating type is defined by the genes present at the MAT *locus* that can be MATa and MATα according to their respective mating type a or α (Fig.6D). While the MAT *locus* is transcriptionaly active, both HMR and HML *loci* that contain copies of MATa and MATα genes are transcriptionaly repressed (Haber, 1998). This silenced state is maintained by the flanking of HMR and HML *loci* by the E and I silencers that recruit the SIR complex to insure the correct repression of these *loci*, whose derepression would lead to a a/α non-mating cells. Nevertheless, the silencing of HMR and HML *loci* is respectively constrained by $tRNA^{thr}$ and CHA1 genes acting here as boundary elements, because their expression blocks SIR-mediated silencing (Donze and Kamakaka, 2001). Other boundary element, named Sub-Telomeric Anti-silencing Regions (STAR) have been initially described as elements that block the spread of telomeric silencing at yeast telomeres. These elements are located between X and Y' repeats and they consist in the repetition of Tbf1 and Reb1 binding sites. The role of these proteins in the anti-silencing activity of STARs has been confirmed since their attachment by the heterologous Gal4 DNA binding domain exhibits a strong boundary activity. In CHO cells, STAR elements were described as blocking the spread of HP1-mediated silencing when located between HP1 binding sites and a reporter gene (Otte et al., 2007).

Boundary proteins have also been described in yeast and mammals. For example, the acidic or proline-rich activation domains of many transcription factors such as CTF1 or BRCA1 have been shown to act as boundary protein in yeast without directly activating the transcription (Fourel et al., 2001). In addition, the Cse1 or Los1 nucleoporins also show a boundary activity that prevents the spread of yeast heterochromatin when tethered to the Gal4 DNA binding domain, and that protects the downstream gene from telomeric silencing (Ishii et al., 2002).

Interestingly, some elements like the CTCF-binding HS4 insulator have been reported to display the features of both insulators and boundaries, making unclear the frontier between them. For example, the HS4 sequence has been shown to protect the chicken β-globin *locus* from the effects of upstream folate receptor gene enhancer or those of the condensed chromatin that separates them (Bell and Felsenfeld, 1999; Chung et al., 1993) (Fig.6B). The insulating sequence between Igf2 and H19 *loci* has also been shown to contain four CTCF binding sites in mouse and seven in Human (Szabo et al., 2000). On the other hand, the possible ability to form functional DNA loop domains by tethering to the nucleolar protein nucleophosmin (Yusufzai and Felsenfeld, 2004), as well as its involvement to prevent chicken telomeric position effect are consistent with boundary activity (Rincon-Arano et al., 2007). Finally, CTCF has been also associated with repression of transcription (Arnold et al., 2000), like at the Sin3A *locus* in NIH3T3 cells by involving histone deacetylases (Lutz et al., 2000). Thus, the effects of this protein remain difficult to classify, and they may depend on the genomic context or on the presence of other proteins.

I.3.4 The transcription factor CTF1.

The transcription factor CTF1 (also named NF1-C1) belongs to the family of CTF/NF1 transcription factors that contains four sub classes including NF1-A, NF1-B, NF1-C and NF1-X (Kruse et al., 1991; Rupp et al., 1990). Most of these genes have been described in many vertebrate species from *Xenopus* to Human (Gronostajski, 2000). CTF/NF1 transcription factors were firstly described as activating viral promoters such as the herpes virus thymidine kinase promoter, and DNA replication by recruiting the viral DNA polymerase at the adenovirus and SV40 origins of replication (Armentero et al., 1994; Muller and Mermod, 2000). Moreover, CTF1 was also described to activate cellular promoters like the murin α- and β-globin promoters (Cohen et al., 1986; Jones et al., 1985), and to mediate TGF-β–regulated transcriptional activation in NIH3T3 cells (Alevizopoulos et al., 1995; Alevizopoulos and Mermod, 1996).

I.3.4.1 Structure of CTF1.

The NF1C gene encodes seven CTF isoforms by alternative splicing. The CTF1 transcription factor has a bipartite structure composed of 499 amino-acids in length (Fig.7). The CTF1 DNA binding domain (a.a. 1-120) permits to CTF1 to bind as a dimer onto TTGGC(N5)GCCAA and related sequences (Hennighausen and Fleckenstein, 1986; Nagata et al., 1983; Santoro et al., 1988), although it may also bind as monomer to TTGGC or GCCAA sequences with a lower affinity (Meisterernst et al., 1988). Its transactivation domain (a.a. 399-499) called Pro for its richness in proline residues contains two sites of interaction with the histone H3 (Muller and Mermod, 2000) : the main H3 interaction domain (TRD), which spreads from a.a. 486 to 499 and binds preferentially the histone variant H3.3 (Ferrari et al., 2004), while the secondary H3 interaction domain is positioned between a.a. 399 and 438 (Alevizopoulos et al., 1995). The TRD domain (for TGF-β responding domain) is also involved in gene transcription activation induced by TGF-β in fibroblasts (Alevizopoulos et al., 1995).

I.3.4.2 Transcription activation and chromatin remodeling activity induced by CTF1.

CTF1 Pro domains is involved in transactivation since its deletion abolishes transactivation in *Drosophila*, mammalian and yeast cells (Chaudhry et al., 1998; Kim and Roeder, 1993; Mermod et al., 1989), and Pro was shown to interact with both TFIIB and TBP in vitro (Kim and Roeder, 1994). Moreover, the presence in Pro of a single heptapeptide repeat (PTSPSYS) similar to that of CTD repeats present in the C-terminal domain of RNA-polymerase II has suggested an interaction with the transcription machinery (Meisterernst et al., 1989; Xiao et al., 1994). A second mechanism proposed for CTF1-mediated transcription activation involves its interaction with nucleosomes. Studies have suggested that histone H1 was able to bind to the consensus CTF1 binding sites, and that transcription might be activated by direct displacement of histones by NF1 members at such sites (Gao et al., 1998; Ristiniemi and Oikarinen, 1989). Two-hybrid assays showed that Pro binds histones H3 and H4 in NIH3T3 cells and *in vitro* (Alevizopoulos et al., 1995), and another study showed that CTF1 is involved in guiding the BRG1 histone remodeling complex to the CSF1 promoter (Liu et al., 2001), leading to chromatin remodelling.

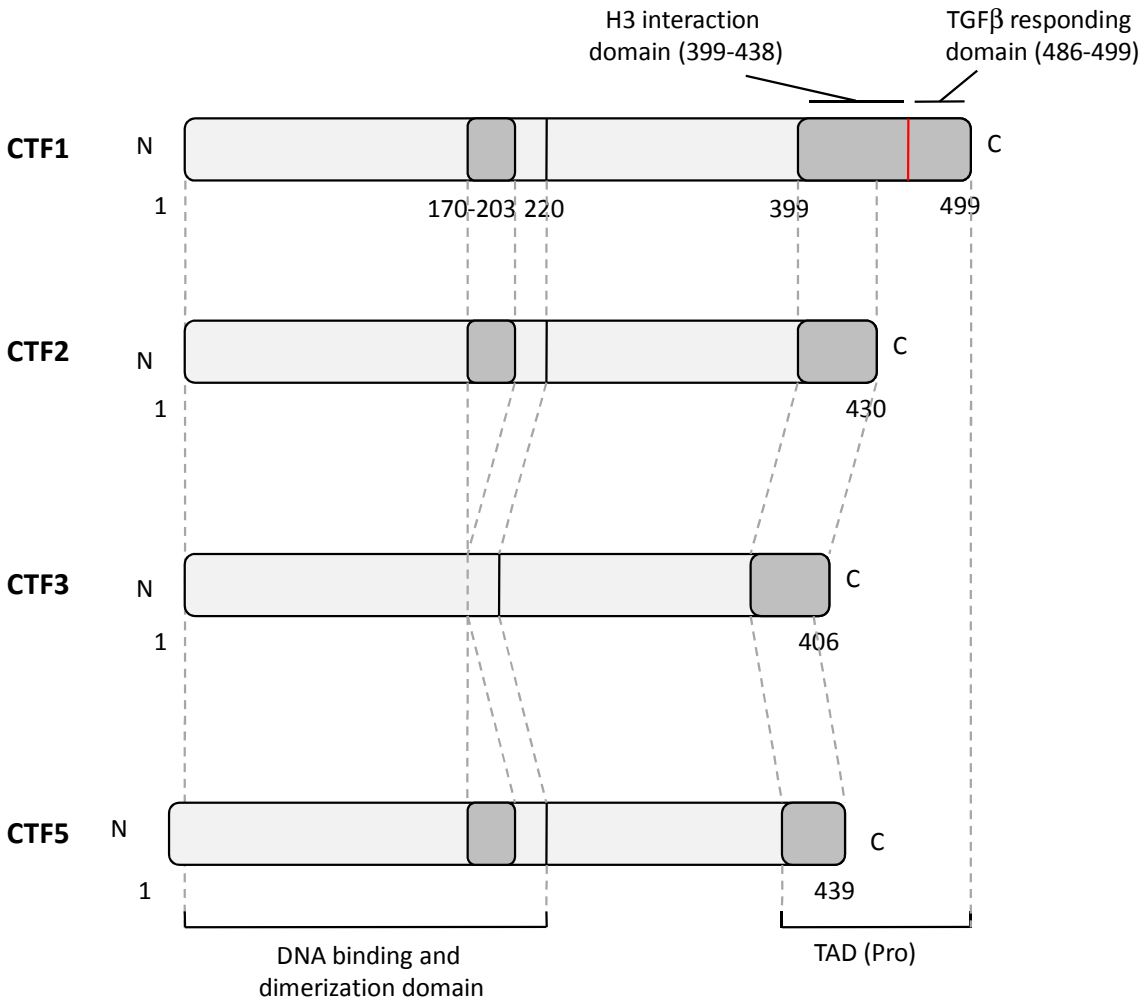


Figure 7. CTF/NF1C isoforms and their functional domains.
Seven isoforms resulting from alternative splicing of CTF/NF1C gene have been described. In a general manner, CTF isoforms have a bipartite structure including the positively charged DNA binding and dimerization domain, and a second domain involved in gene activation and/or histone binding. DNA binding activity is carried by the region located between a.a 180 and 220, but the region absent in CTF3 (a.a 170-203, left grey) is dispensable for high affinity binding. CTF1 is the isoform displaying the complete proline rich transactivation domain (right grey) composed of a histone H3 interaction domain (a.a 399-438) and a TGF-β responding domain (a.a 486-499) binding also to histone H3. The sequence PTSPSYS (a.a 460-467) similar to CTD repeats is shown in red. In comparison, other isoforms depicted here possess only a shortened transactivation domain that includes only the first histone H3 interacting domain and exhibit a weaker transcription activation.

The histone remodeling activity of CTF1 has been also studied in the context of gene expression regulation. Indeed, chromosomal position effect is a current problem for transgenesis since many integrated transgenes become silenced over time. Thus, insulating a transgene from its environment would avoid silencing by the spread of neighboring heterochromatin. When fused to the yeast Gal4 DNA binding domain (DBD), CTF1 Pro domain shows a high unsilencing activity in mammalian and yeast cells (Ferrari et al., 2004; Fourel et al., 2001; Pankiewicz et al., 2005). This unsilencing activity has been attributed to its main H3 binding domain, as it is required for this activity (Pankiewicz et al., 2005). Moreover, experiments in yeast indicate that the Gal-Pro fusion can protect a gene against telomeric silencing, and that this activity requires the ability to interact with H3 histones. This effect was shown to restore histone acetylation on the *locus* protected from telomeric silencing (Ferrari et al., 2004), indicating that Gal-Pro acts as a strong artificial boundary protein in *S.cerevisiae*. Nevertheless, whether Gal-Pro or the entire CTF1 protein may also exhibit unsilencing activity at mammalian telomeres is not known.

I.4. Aim of this work.

The control of transgene expression is a common problem in biotechnological applications such as in gene therapy, since the *locus* of transgene integration is often subject to silencing and chromosomal position effect. Nonetheless, controlling transgene expression should not lead to modification of the expression of neighboring genes, and therefore avoiding the recourse to strong transcriptional activators or enhancers may be advantageous. As described above, CTF1 displays both an unsilencing activity in mammals and a boundary effect against telomeric silencing in yeast that seem to be independent of its transcriptional activity. However, little is known about the potential ability of CTF1 to remodel chromatin domains in mammals, nor about the modifications that may be mediated by CTF1 on a silent chromatin environment in human cells.

Telomeric environment is a well characterized system in which heterochromatin may spread from a defined direction, although mammalian telomeric heterochromatin features remain mostly unknown. In order to assess the ability of CTF1 to separate chromatin domains in human cells, the first step of this work consisted in the development of a two-reporter

system located at human telomere that would permit to measure the boundary activity of Gal-fusion proteins against telomeric position effect, in a quantitative fashion, and that would allow the study of potential chromatin modifications associated with a boundary activity. Such system would allow to assess whether CTF1 or fusion derivatives mediate a protection against heterochromatin, when positioned between a telomere distal reporter gene and the telomere, whereas another telomere-unprotected transgene on the telomere side would remain silenced by telomeric position effect.

Telomeric silencing is mediated by propagation of SIR-dependant histone deacetylation in yeast cells, but no such spread has been demonstrated in mammalian cells as yet. The presence of Sirtuin proteins at mammalian telomeres suggests that histone deacetylation may occur in TPE, but the presence of other histones marks suggests that several chromatin modifications might be involved in TPE. However, how this histones marks lead to TPE in such cells remains mostly unknown. Then, the two-reporter system described here may be used to compare histones marks present at telomeric chromatin with those at an internal position in the chromosome in order to determine which histones marks are involved in telomeric heterochromatin. On the other hand, the comparison of the histones marks present at a *locus* unprotected from telomere influency with those present at a *locus* insulated from the telomere, within the same clonal population, would allow to understand how transgenes can be protected from the silencing mediated by a close constitutive heterochromatin, and which histones marks may be involved in the establishment of a chromatin boundary.

II. DEVELOPMENT OF NEW TOOLS FOR THE STUDY OF BOUNDARY PROTEINS.

II.1 Introduction

The study of boundary proteins has involved the integration of one or several reporter genes at a position close to the telomere. In yeast, genes inserted near the telomere by homologous recombination were prone to a strong telomeric silencing. Unfortunately, homologous recombination remains difficult in mammalian cells and chromosomal recombination that occurs frequently in cancerous cell lines makes it difficult to target a specific *locus* of integration. This problem has been mainly resolved by the use of plasmids containing telomeric repeats that allow transgenes integration at human telomeres as shown in the study of Baur *et al.* (2001). Although this study showed that transgenes inserted near HeLa cells telomeres were prone to telomeric silencing (Baur et al., 2001), that of Kim *et al.* (1999) suggested that telomeres are a *locus* of high and stable gene expression in CHO cells (Kim and Lee, 1999), leaving a doubt about the presence of heterochromatin at mammalian telomeres. Consequently, comparing telomeric silencing in several mammalian cell lines may be a first step before to study the effects of boundary proteins. Moreover, the widespread use of CHO cells in biotechnologies makes the determination of elements controlling gene expression in this cell type particularly useful. In the work presented here, this step was firstly realized by comparison of transgene expression near HeLa telomeres with that of transgenes inserted at CHO telomeres, to identify the most suitable cell type for further experiments.

The two-reporter system described by Ferrari *et al.* (2004) in yeast was composed of the URA3 and TRP genes which are necessary to cell survival. Boundary activities were measured in function of cell survival, positively influenced by URA3 silencing and TRP expression when Gal-fusion proteins bind between the reporter genes. Nevertheless, such genetic gene selection systems often mediate cytotoxic effects, which may in addition affect chromatin modifications observed after binding of a boundary protein between the reporter genes. Thus, such system is not optimal to extrapolate results to a normal chromatin regulation, and these reporter genes are not suitable for use in mammalian cells. Nevertheless, the use of two reporter genes system remains a clever strategy to detect boundary activities, and no equivalent reporter system has yet been designed for use in mammalian cells. Indeed, few studies have analyzed telomeric position effect or elements protecting from telomeric silencing in mammalian cells using only one reporter gene, which was not sufficient to compare the telomere-protected side with silenced one nor to address potential variations in

cellular silencing effects (Baur et al., 2001; Koering et al., 2002; Rincon-Arano et al., 2007). Consequently, instead of using reporter genes that are indispensable for cell survival, gene encoding fluorescent proteins were used to observe boundary activities and associated chromatin modifications in living cells without cytotoxic effects, which may be closer to a normal chromatin mediated regulation.

Nevertheless, the use of fluorescent proteins is often prone to the cross detection of their fluorescent spectra at high fluorescence levels, which involves to choose judisciouly each fluorescent protein in function of its wavelength and to optimize their detection to analyze distinct wavelength ranges. On the other hand, the analysis of telomeric boundaries requires that telomeric transgenes should be sufficiently expressed to be easily detected, while a use of regulatory elements such as enhancers may avoid silencing effects from the telomere. Moreover, both reporter genes must display similar fluorescence levels. Thus, several plasmid constructions conciliating telomeric integration and the two-reporter systems had to be constructed and assessed for their suitability in the study of boundary proteins.

II.2 Material and methods.

II.2.1 Cell culture.

The *E.coli* strain DH5α was cultivated at 37°c on LB agar plates or in LB liquid medium with agitation. Transformed bacteria were cultivated with addition of ampicillin at the concentration of 100µg/mL. HeLa Tet-off cells (Clontech) and CHO Tet-off cells (Clontech) were cultivated at 37°c and 5% CO2 in DMEM-F12 (Gibco) with 10% foetal bovine serum (Gibco).

II.2.2 Vector constructions

The pBX-R and pBX-NR plasmid vectors were kindly provided by J. Baur (Baur et al., 2001). For luciferase analysis, the plasmids remained identical to that used in the study of Baur *et al*.

The pGE plasmids and derivatives were obtained by cloning telomeric repeats from pBX-R in a pBS2-SKP plasmid. Puromycin resistance gene, including its CAG promoter was inserted in the previous plasmid giving the pBS-R-CAGPuro plasmid. Reporter genes DsRed

and EGFP under the control of the complete CMV promoter were subcloned in a pBS2-SKP plasmid and further inserted in pBSR-CAGPuro plasmid, giving the pGE plasmid. Plasmids carrying DsRed and EGFP under the control of minimal CMV promoter (pGEmin and pGE2min) were obtained by PCR amplification of reporter genes, excluding the CMV enhancer and were inserted in pBSR-CAGPuro.

The Gal4 binding sites were obtained by annealing of each oligonucleotides (gatccgggtcggagtactgtcctccgactgc and gatcgcagtcggaggacagtactccgacccg) generating BamHI cohesive extremities, phosphorylated and multimerized by ligation. The cassette of four GAL4 binding sites were inserted in the unique BamHI site in pGE or through AscI and Avr2 binding sites in plasmids deleted from CMV enhancer, giving respectively pGE-Gal4, pGEmin-Gal4 and pGE2min-Gal4. Integrity of Gal4 binding sites and PCR amplified transgenes was confirmed by sequencing.

All control plasmids without telomeric repeats were obtained by cleaving the repetitions of the previous plasmid with XbaI and NotI, filling-in and religating.

II.2.3 Transfections and selection of stable clones.

II.2.3.1 Transfections of pBX-R and pBX-NR in HeLa and CHO cells.

HeLa and CHO cells grown in 6-wells plates and were transfected with 2µg of DNA and 6µL of Fugene 6 reagent (Roche Diagnostics) per well according to the commercial protocol. Before transfection, pBX-R and pBX-NR plasmids were respectively linearised with PvuI and NotI or PvuI alone. Selection by puromycin was performed for three weeks at the concentration of 2µg/mL for Hela cells and 5µg/mL with CHO cells. Stable clones were isolated by limiting dilution and puromycin selection was stopped at least two weeks before analysis.

II.2.3.2 Transfections of two reporters plasmids.

Transient transfections with pGE-Gal4, pGEmin-Gal4 or pGE2min-Gal4 with Gal-VP16 or Gal-DBD encoding plasmids were performed using circular plasmid, with the same protocol as described above, using a ratio reporter plasmid and GalVP16 of 10:1.

Stable transfections were processed using Fugene 6 commercial protocol and pGE-Gal4, pGEmin-Gal4 or pGE2min-Gal4 plasmids linearised with NotI and ApaLI, or with

ApaLI only for their respective controls without telomeric repeats, and selected for three weeks at the concentration of 2µg/mL of puromycin. Transfections of Gal-fusion proteins expression plasmids and the BFP expression plasmid were performed 48h before analysis using 4µg of circular DNA and 6µL Fugene 6 reagent per well according to the commercial protocol.

II.2.4 Luciferase assay

Cells grown on 6-weels plates until a confluence of 90% and were washed with PBS. Cells were lysed with 200 µL per well of lysis buffer (3.03g/L Tris, 0.695g/L CDTA, 10% glycerol, 1%t triton, 2mM DTT, pH=7.8) for 20 minutes. Lysate was centrifuged at 12000 rpm for 10 minutes and 20µL of supernatant were added to 100µL of luciferase assay reagent (20mM tricine, 0.1mM EDTA, 1.07mM (MgCO3)4Mg(OH)2.5H2O, 2.67mM MgSO4, 33.3mM DTT, 270 µM Coenzyme A, 470 µM luciferin, 530µM ATP). Luciferase activity was immediately measured in Microlumat Plus luminometer (EG&G Berthold).

The relative quantity of protein in each sample used to normalize the luciferase activity was measured by addition of 5µL of the same lysate to 195µL of Bradford reagent (40µL Biorad protein assay, 155µL H2O). After 10 minutes of incubation at room temperature, the luminescence was measured at 595nm.

II.2.5 Southern blotting.

II.2.5.1 Extraction of genomic DNA.

Cells grown on 6-weels plates until a confluence of 90% and were washed with PBS. In each well 300µL of lysis buffer (0.5% SDS, 0.1M NaCl, 50mM Tris, 5mM EDTA, 0.1 mg/mL proteinase K) was added and incubated for 16 hours. The lysate was precipitated by addition of 300 µL of isopropanol and centrifuged at 14000 rpm at 4°c for 30 minutes. Precipitated DNA was resuspended in 100µL of Tris-EDTA buffer. The concentration was determined by O.D (260nm).

II.2.5.2 DNA electrophoresis and transfert.

The DNA (10µg) was digested by StuI (100 units) at 37°c over night in a volume of 100µL containing the commercial buffer and 10% of BSA. Restriction fragments were separated by electrophoresis on agarose gel (TAE - 0.8% agarose). After migration, DNA was

depurined by immersion in HCl 0.25N for 10 minutes, and washed with distilled water. Then, DNA was transferred on a Hybond-N+ nylon membrane (Amersham) by capillarity during the 16 hours using a solution of NaOH 0.4M. The membrane was washed with 2xSSC, and prehybridized in CHURCH (NaH2PO41 10mM, Na2HPO4 387.5mM, SDS 7%) 1h at 65°c with denatured salmon sperm DNA (100 µg/mL).

II.2.5.3 Generation of the probe.

The DNA used to generate the probe was the luciferase fragment isolated from the pBX plasmid after digestion with XbaI and EcoRI. The luciferase fragment was purified and used as matrix for the High prime kit (Roche) according to the commercial protocol. The High prime polymerase generates single stranded DNA using random primers, and incorporates ^{32}P-dCTP. Then the radioactive probe was purified on Microspin-G50 sephadex column (Amersham) before a next use.

II.2.5.4 Hybridization and revelation.

After denaturation for 5 minutes at 95°c, the radioactive probe was added in the prehybridation buffer. Hybridation was performed over night at 65°c. Then, the membrane was washed with 2xSSC-1% SDS for 10 minutes at 65°c, and 0.2x SSC-0.1% SDS for 20 minutes at 65°c in order to increase the stringency. The membrane was exposed during two days to the storage phosphor screen (Kodak) and the signal was read on Storm analyser (Amersham Pharmacia Biotech).

II.2.6 Flow cytometry.

Fluorescence analysis was performed on either the FACS Calibur (Dickinson-Becton) with settings of 520V on the SSC channel, 380V on GFP dedicated laser and 550V on DsRed dedicated laser, or on the FACS Cyan (Dakocytomation) with the settings of 550V on the SSC channel, 530V on the GFP and DsRed dedicated laser and 650V on the BFP channel. Cells expressing high levels of BFP were considered starting with 10^2 AFU.

II.3. Results.

II.3.1 Silencing of telomere proximal genes in mammalian cells.

II.3.1.1 Comparison of transgene expressions at mammalian telomeres

While Kim *et al.* (1999) did not insure the targeting of transgene at CHO telomeres, Baur *et al.* (2001) used either a linearized plasmid containing the luciferase reporter gene adjacent to 1.6Kb of telomeric repeats (pBX-R), or a control plasmid containing the luciferase reporter without telomeric repeats (pBX-NR). After Puromycin selection and isolation of clones with stably integrated transgenes, telomeric position can be verified by Southern blot and/or Fluorescent *in situ* Hybridization (FISH). Generally, clones transfected with the plasmid without telomeric repeats display internal chromosomal insertions while those transfected with the repeats will integrate the luciferase reporter at a telomeric position. This is supposed to be the consequence of single crossing-over integrations that lead generally to cell death when telomeric repeats are not in the plasmid vector, whereas their presence would allow cell survival by creating a new telomere wen using the construct containing the telomeric repeats. However, the targeting of the reporter construct at a preexisting telomeric region cannot be excluded (Baur et al., 2001; Kilburn et al., 2001). Stable clones transfected with the telomeric repeats-containing plasmid display high frequencies of telomeric insertions, unlike those transfected without telomeric repeats, which are used as internally inserted control transgenes. In telomeric clones, the telomeric position effect can result in a 10-fold lower luciferase expression as compared to transgene insertion at an internal *locus*.

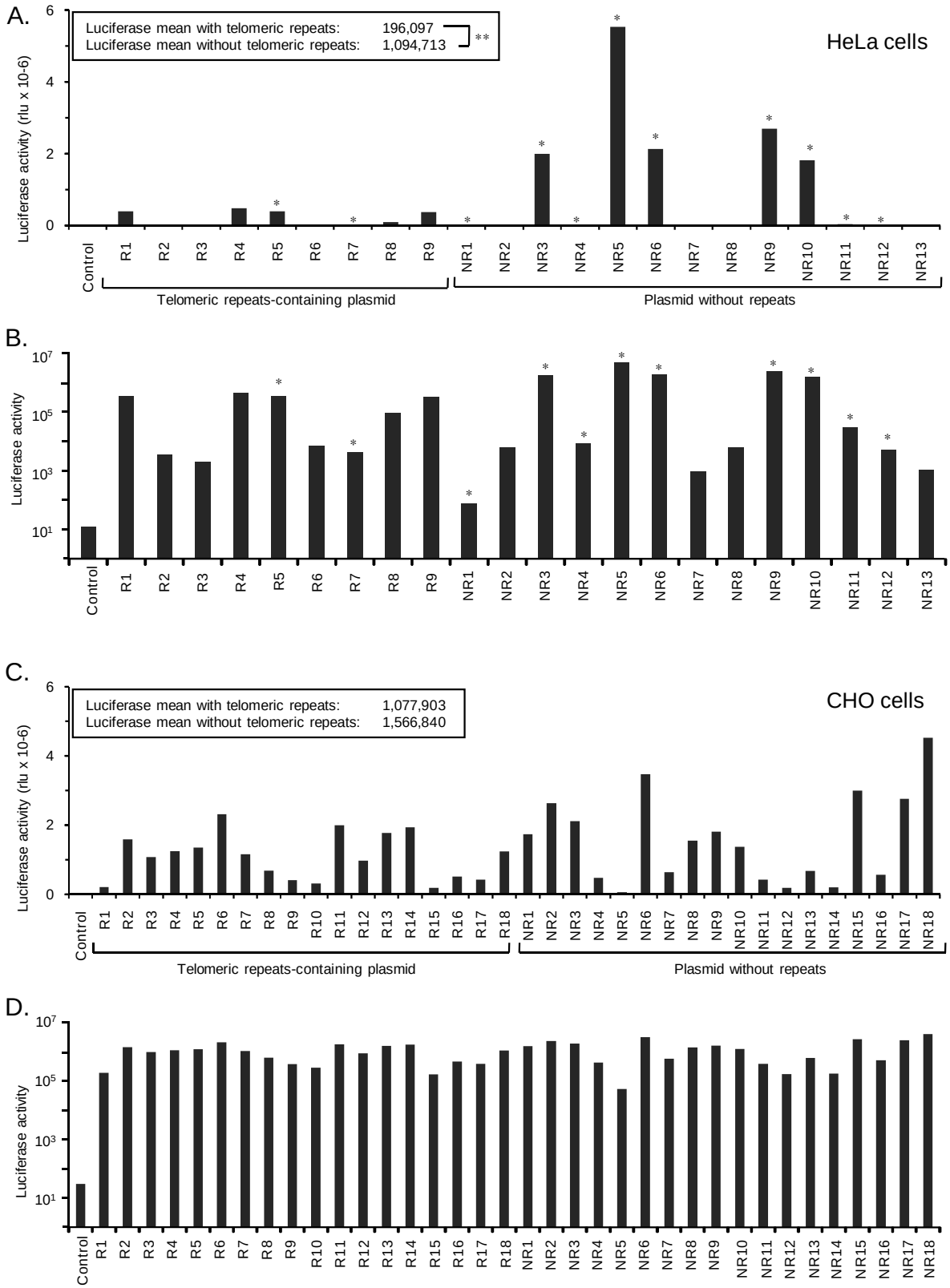
II.3.1.2 Telomeric position effect in HeLa and CHO cell lines.

To compare telomeric position effect of human cells with that of CHO cells, the HeLa cell type was used for its documented frequent rate of telomeric integrations (Kilburn et al., 2001). Both cell types were transfected as described by Baur *et al.* (2001), antibiotic-resistant cells having stably integrated transgenes were selected, and monoclonal populations were isolated and expanded. Finally, telomeric insertions were verified by Southern blotting. Internal insertions were differentiated from telomeric insertions by the size of the band observed. Indeed, by digestion of genomic DNA with a restriction enzyme cutting in a single site inside the pBX-R and pBX-NR plasmids, a band is detected whose size depends on the position of a second restriction site in the chromosome, but the fragment size remains constant

for cells of a given clone. In contrast, when luciferase is adjacent to the telomere whose size can vary from cell to cell, DNA fragments with heterogeneous sizes appear as a smear (Baur et al., 2001). Although internal bands were easier to detect than telomeric smears, Southern blotting assayed in 18 HeLa clones confirmed a frequency of internal insertions of 77% for the clones transfected without repeats, but only 22% for those transfected with the telomeric repeats-containing plasmid (data not shown). Moreover, internal insertions were frequently associated with higher reporter expression in this cell line (Fig.8). However, for the 42 CHO clones analyzed, frequency of internal integrations was similar for clones transfected with the plasmid containing telomeric repeats as compared to those transfected without them. Moreover, internal integrations did not correlate well with high luciferase activities.

Luciferase activities were measured in 22 HeLa clones and 36 CHO clones. In Hela cells, clones transfected with the telomeric repeats-containing plasmid displayed a significant lower luciferase activity mean despite the presence of clones inserted at an internal *locus* within this category (Fig.8 A, B). Taken together, these clones display a 5-fold lower mean luciferase activity ($P<0.05$; Student's *t*-test) than clones transfected without telomeric repeats, as expected from a putative telomeric position effect. Moreover, this ratio increases up to 8-fold ($P<0.05$; Student's *t*-test) if potential telomeric clones among those transfected with telomeric repeats are compared with internal integrated clones among those transfected without repeats. In CHO cells, clones transfected with the telomeric repeats-containing plasmid displayed only a 1.5-fold lower mean luciferase activity as compared to clones transfected without repeats (Fig.8 C, D), and the similar frequency of internal integrations in both categories of clones make difficult to consider that telomeric integrations occur among the clones transfected with telomeric repeats.

Figure 8. Evidence for the telomeric silencing in HeLa and CHO stable clones.
Luciferase assay in 22 HeLa stable clones (A: linear scale, B: logarithmic scale) and 36 CHO stable clones (resp. C and D). The R-labelled clones have been transfected with the telomeric repeats containing plasmid (pBX-R) and the NR-labelled clones with the control plasmid without repeats (pBX-NR). Clones transfected with the telomeric repeats containing plasmid show a 5-fold lower mean activity of luciferase in HeLa cells, and only 1.5-fold in CHO cells. Controls are relative to untransfected cells. (*) Clones whose internal insertion was confirmed by Southern blot. (**) $P<0.05$; Student's *t*-test. Rlu: relative light units.


A.
Luciferase mean with telomeric repeats: 196,097
Luciferase mean without telomeric repeats: 1,094,713
**
HeLa cells
Luciferase activity (rlu x 10-6)
Control
R1 R2 R3 R4 R5 R6 R7 R8 R9
NR1 NR2 NR3 NR4 NR5 NR6 NR7 NR8 NR9 NR10 NR11 NR12 NR13
Telomeric repeats-containing plasmid
Plasmid without repeats
B.
Luciferase activity
C.
Luciferase mean with telomeric repeats: 1,077,903
Luciferase mean without telomeric repeats: 1,566,840
CHO cells
Luciferase activity (rlu x 10-6)
Control
R1 R2 R3 R4 R5 R6 R7 R8 R9 R10 R11 R12 R13 R14 R15 R16 R17 R18
NR1 NR2 NR3 NR4 NR5 NR6 NR7 NR8 NR9 NR10 NR11 NR12 NR13 NR14 NR15 NR16 NR17 NR18
Telomeric repeats-containing plasmid
Plasmid without repeats
D.
Luciferase activity

The frequency of telomeric integrations observed in HeLa cells is consistent with that of the 70% noted by Kilburn *et al.* (2001) and it may explain the significant decrease of luciferase activity observed in HeLa cells, although HeLa cells might be also particularly prone to TPE (Kilburn et al., 2001). Considering these evidences, the HeLa cell line appears as the most suitable for a study in a context of telomeric silencing because of its facility to insert a transgene in a telomeric position and its better silencing of telomeric transgenes. Thus, this cell line was chosen for the following experiments, while the CHO cell line appears to be inappropriate for the study of telomeric silencing.

II.3.2 Construction of two reporters system for the study of boundary proteins.

II.3.2.1 Plasmid constructions designed for the study of boundary proteins.

The strategy elaborated to observe the boundary effect of CTF1 against the telomeric silencing consists in analyzing the expression of two reporter genes in a telomeric context using the mechanism of telomeric insertion described previously. Due to the telomeric position, both reporter genes might be silenced, and binding a boundary protein between them would protect the telomere distal gene from the silencing, while leaving the telomere proximal reporter gene silenced. Nevertheless, instead of CTF1 binding sites, Gal4 binding sites were firstly preferred to allow the possibility of analyzing different Gal-fusion proteins within the same clone, such as Gal-VP16 supposed to activate transcription for both reporter genes in spite of the telomeric silencing. In these vectors, telomeric repeats were obtained from the plasmid previously described, but fluorescent proteins (GFP and DsRed) were preferred for their direct and easier detection in single living cells, whereas the luciferase needs an enzymatic assay on a pool of cells. In the perspective to obtain (1) an easy and frequent telomeric silencing and (2) to distinguish effectively unsilencing effects, three plasmid vectors were tested in this study to determine the most adapted promoter and the best orientation of the reporters.

Firstly, just pGE-Gal4 plasmid was constructed (Fig.9 A), in which DsRed and GFP reporter genes are controlled by the same promoter (CMV) to maintain similar expression levels, and they are positioned in an opposite direction trying to exclude the possibility of direct transcription activation by Gal-fusion proteins because of their proximity to the promoter. Cell lines were generated and characterized for telomeric integration. However, inappropriate results obtained with this plasmid (see below), and two other vectors were

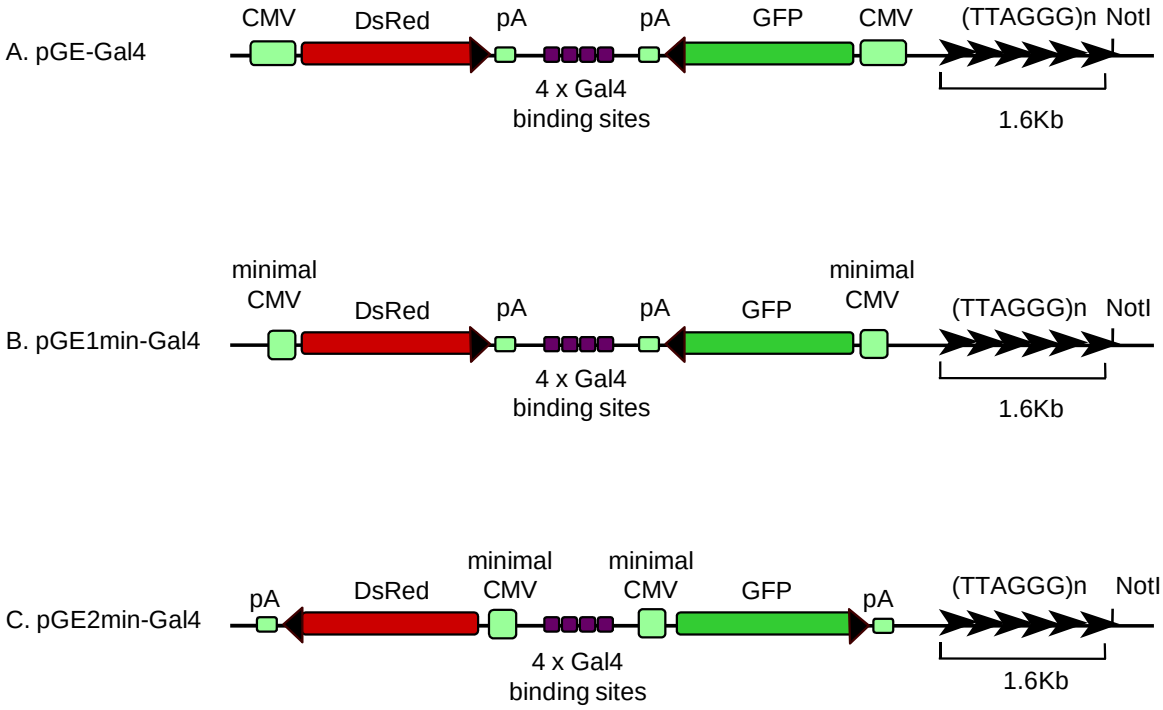


Figure 9. The three vectors designed to observe potential boundary effects of Gal-fusion proteins.
(A) pGE-Gal4 construction is composed of the two reporter genes (GFP and DsRed) controlled by the strong CMV promoter, including its enhancer. GFP and DsRed are oriented in order to move the promoter away from the Gal4 binding sites. (B) pGEmin-Gal4 plasmid is similar to the precedent, except that DsRed and GFP reporters are controlled by a CMV promoter deleted of its enhancer (minimal CMV). (C) pGE2min-Gal4 plasmid is constructed by reversing of the DsRed and GFP reporters, always under the control of the minimal CMV promoter. All of these constructions contain 1.6Kb of telomeric repeats to facilitate the telomeric insertion in the genome, and are preceded by the puromycin resistance gene (not shown). Control plasmids (not shown) are similar except for their deletion of telomeric repeats.

constructed (Fig.9 B and C). Consequently, the pGE-Gal4 plasmid was derived in the pGEmin-Gal4 plasmid in which GFP and DsRed reporters are controlled by the CMV promoter deleted from its enhancer (minimal CMV), which may facilitate the silencing of reporter genes while allowing a better effect of Gal-VP16 transcriptional activator. The reverse orientation of the GFP and DsRed reporter genes was also tested with the pGE2min-Gal4 plasmid in which the reporter genes are divergently transcribed from the minimal CMV promoter.

II.3.2.2 Assessment of the most appropriate plasmid construct for the study of boundary proteins.

Firstly, the basal expression of reporter genes was tested through co-transfection of the plasmid reporter construction with a control plasmid (Fig.10 D,G,J), whereas transcription activation without epigenetic interference was tested by the co-transfection of the same plasmid reporter constructs with a Gal-VP16 encoding plasmid (Fig.10 F,I,L) or with a Gal-DBD control vector (Fig.10 E,H,K). Among the three constructs tested, pGE-Gal4 show the highest basal expression of GFP and DsRed (Fig.10 D,G,J), and the activation by Gal-VP16 induces only a 1.2-fold increase of high expressing cells (Fig.11), suggesting that DsRed and GFP are already expressed at their maximal level of expression. In contrast, the two other constructions have a lower basal expression of reporter genes, but comparison of Gal-VP16 activation for these two constructions shows that only pGE2min-Gal4 can increase significantly the expression of reporter genes, which is comforted by the fact that activation by Gal-VP16 is lost when Gal4 binding sites are removed from the plasmid construction (Fig.11).

Stable clones isolated from pGE-Gal4 transfection confirmed also the high basal expression of the reporter genes and the difficulty to activate transcription with Gal-VP16 (data not shown). Moreover, such clones expressed either more DsRed or more GFP, but rarely the two reporter genes with the same level, which might be the consequence of a competition of RNA-polymerases transcribing in opposite directions. In contrast, clones isolated from pGEmin-Gal4 and pGE2min-Gal4 transfections expressed generally both reporter genes at the same level and they were easily silenced. Thus, for each of these plasmid constructs and for their respective repeats-deleted controls, 25 clones were analyzed to assess the suitability of their reporter gene expression in regard to the study of boundary proteins.

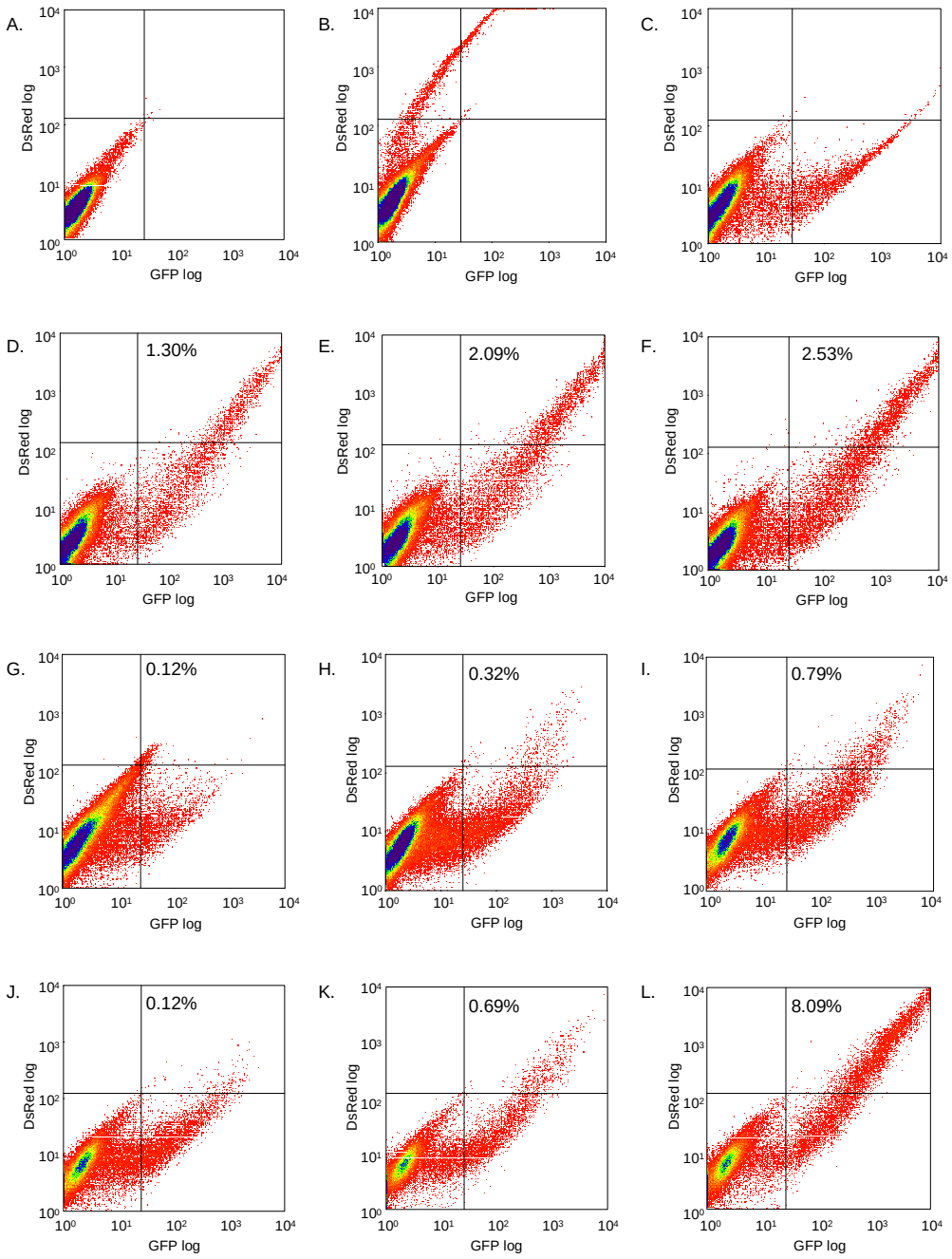


Figure 10. Basal or activated expression of the DsRed and GFP reporters.
FACS analysis of DsRed and GFP fluorescences in HeLa cells transiently transfected with the three reporter plasmids shown in Fig.9. Cells expressing no fluorescent protein, DsRed or GFP are respectively shown panel (A, B, C). (D, E, F) pGE-Gal4 plasmid respectively co-transfected with a control plasmid, a Gal-DBD expression plasmid or a Gal-VP16 expression plasmid. Cells carrying the pGEmin-Gal4 and pGE2min-Gal4 are respectively shown in (G, H, I) and (J, K, L). Quadrant regions are determined on the basis of untransfected cells (A) in order to obtain at least 99% of autofluorescent cells in the bottom left region. High expressing cells are ranged in the R2 region (top right). Percentages are function of the proportion of cells in the R2 region compared to the total sample. Fold increase of the R2 percentages in GalVP16 transfection relative to the GalDBD value is shown Fig.11. Each analysis results from the analysis of 100.000 cells 3 days *post*-transfection.

Transfection of each clone with Gal-VP16 and BFP expressing plasmids (ratio 1:1) permitted to compare by FACS analysis the cells expressing high levels of BFP with those expressing lower levels of BFP, and therefore to exclude from the study the clones displaying the following criteria :

- Heterogeneous DsRed and GFP expression, because of a probable multiple insertion and/or an imperfect clonal isolation.

- Clones with a high basal expression of GFP and DsRed which may result from internal insertion.

- No activation of DsRed and/or GFP by Gal-VP16. Such clones may be either very difficult to unsilence, or may be deleted of one or both reporter genes.

- Clones expressing DsRed at higher levels than GFP, because a boundary effect in these clones would be partly or totally hidden.

Among the remaining clones, four were selected for their low expression of reporter genes as expected from a telomeric silencing (Fig.12 A), while clones displaying higher levels of reporter genes were also isolated as negative controls of TPE (Fig.12 B). In order to control that reporter gene expression in these clones is well resulting from their position in regard to the telomere, rather than from a preferential expression of one reporter gene, control clones transfected with the repeats-deleted plasmids were also isolated with similar expression levels as in the first category of clones (Fig.12 C).

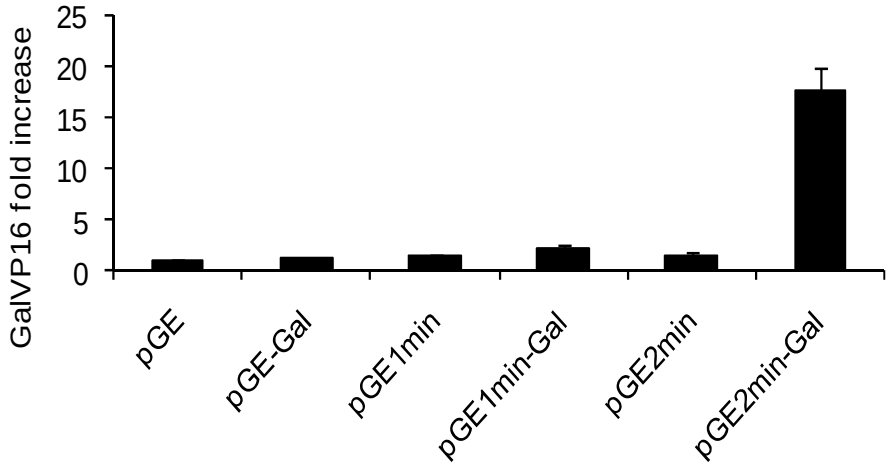


Figure 11. Gal-VP16 activation and specificity of the Gal4 binding sites in the different plasmid constructions.

Values represent the fold-increase of cells expressing high levels of DsRed and GFP (Fig.10. R2 region) when different reporter plasmid constructions are co-transfected with a Gal-VP16 expression plasmid. Values were normalized with the fluorescence values obtained with a GalDBD expression plasmid. 'pGE', 'pGEmin' and 'pGE2min' differ from the three plasmids shown Fig.9 by the deletion of Gal4 binding sites between GFP and DsRed. These three plasmids show no increase by GalVP16, excluding an aspecific activation of Gal-VP16. pGEmin-Gal4 and pGE2min-Gal4 constructions are more activated by GalVP16. As shown in Fig.10, pGE2min-Gal4 shows the best effect. Error bars: SEM on three independent experiments.

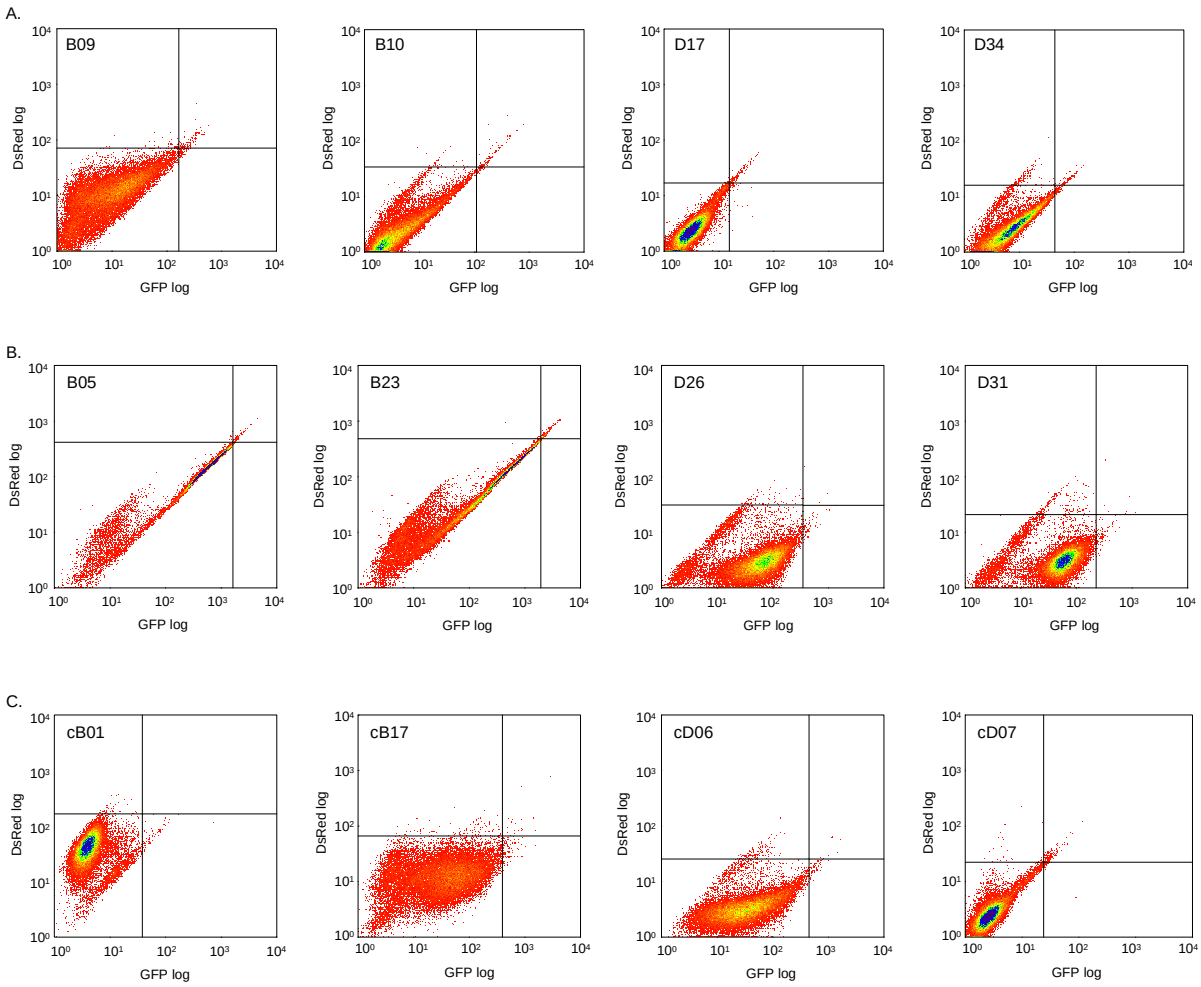


Fig. 12. Pattern of expression of DsRed and GFP for the 12 monoclonal populations selected from stable transfections.

HeLa cells were transfected with the Not1-linearized reporter plasmids as shown in Fig. 9B and 9C, antibiotic resistant cells were selected and monoclonal populations were generated by single cell sorting. Clones were characterized as described in the main text and the GFP and DsRed expression were analyzed by cytofluorometry. The six panels on the left depict clones carrying the plasmid with convergent reporter gene transcription and the six clones on the right hand side have divergent reporter genes. (A) Representative telomeric clones expressing undetectable or low levels of the reporter proteins. (B) Representative telomeric clones expressing detectable levels of the reporter proteins. (C) Representative clones generated from transfections with the plasmid devoid of telomeric repeats, and showing a non-telomeric location by FISH analysis.

Interestingly, both transient and stably transfected cells showed preferential detection of GFP, but only for the lower levels of expression. This preference was observed only when reporter genes were controlled by a minimal CMV promoter, and was also observed for telomeric repeats deleted constructions. Analysis of transient and stable expressions of pGE-Gal4 reporter genes suggest that the strong CMV promoter is inappropriate to obtain a silencing when integrated in the genome, and its lack of activation by Gal-VP16 suggests that DsRed and GFP reporters may be already at their highest level of expression. For these reasons, the pGE-Gal4 construction was less suitable for this study and thus not used in next experiments.

The difficulties to obtain Gal-VP16 activation with pGEmin-Gal4 compared to pGE2min-Gal4 show that the distance between Gal4 binding sites and the CMV promoter is determinant for transcription activation mediated by Gal-VP16. Consequently, the first reporter construct avoids any transcriptional activation by Gal-fusion protein and allows only the detection of chromatin boundary effects. In contrast, the second construct may insure an easier desilencing of reporter genes by activators such as Gal-VP16. In addition, testing the plasmid constructs deleted from Gal4 binding sites excluded an aspecific binding of Gal-VP16 or other Gal-fusion proteins (Fig.11). Consequently, both pGEmin-Gal4 and pGE2min-Gal4 constructions were considered as suitable to analyze the activity of boundary proteins.

II.4. Conclusions.

Silencing of genes at mammalian telomeres was disputed by a few studies showing opposite results. Nevertheless, these studies did not use the same experimental approach. Here, both HeLa and CHO cell lines were analyzed for their telomeric position effect using a plasmid containing telomeric repeats to allow a transgene position close to the telomere. In agreement with Baur *et al.* (2001), the presence of telomeric repeats in the plasmid construction may be associated with telomeric integration since internal integrations were less frequent in the clones transfected with telomeric-repeats. Moreover, the luciferase assay confirms that HeLa telomeres are a *locus* of silencing for neighboring transgenes. Indeed, the comparison of clones transfected without telomeric repeats with those transfected with repeats results in a significant 5-fold decrease in the mean luciferase activity; and up to 8-fold when putative telomeric clones are compared with internal inserted clones, while Baur *et al.* (2001) described a 10-fold decrease. In CHO cells, the silencing associated to telomeric repeats was dubious since it reaches only 1.5-fold decrease in the mean of luciferase activity, which was not statistically significant. Moreover, the similar frequency of internal integrations observed in cells transfected with or without telomeric repeats suggests that this cell line is less prone to telomeric insertion than HeLa cells. This could explain the lower decrease in luciferase activity observed when CHO cells are transfected with telomeric repeats. Then, the low rate of internal integrations observed in HeLa cells after transfection with telomeric repeats-containing plasmid dictated the choice of this cell line as the best candidate to study telomeric silencing. Nevertheless, the one reporter-plasmid described here remains non-ideal for the study of boundary proteins.

Thus, three dual-reporters plasmid constructs carrying the GFP and DsRed reporters were tested here. FACS analyses confirmed that the entire CMV promoter remains stronger to allow an easy silencing of reporter genes inserted near the telomere. In contrast, minimal CMV promoter allows the silencing of reporter genes when located near the telomere, but without difficulties in detecting their unsilencing, as confirmed by GalVP16 induction. FACS analyses suggested that DsRed and GFP are not expressed at the same level in spite of their proximity. Nevertheless, unsilencing of DsRed remains detectable but it suggests that this system would preferentially detect proteins that have a high boundary activity, rather than those allowing only a low expression of DsRed. The use of Gal4 DNA binding sites permits to bind specifically Gal-fusion proteins while avoiding the binding of these proteins to non

specific sequences, as shown by the comparison of constructs with or without Gal4 DNA binding sites co-transfected with GalVP16. Moreover, the use of Gal4 DNA binding sites allow to compare within the same clones - and thus in similar chromatin environments – many Gal-fusion proteins such as the Gal-TRD mutants studied by Pankiewics *et al.* (2005), or many other putative boundary protein. In addition, the boundary effect of other insulating sequences might be also studied provided minimal modifications of these vectors and construction of appropriate reporter cell lines.

Finally, the use of fluorescent proteins like GFP and DsRed permits to analyze boundary elements in single living cells, and as well to quantify the proportion of cells in which this phenomenon occurs, where luciferase measure could quantify only a average value on several thousand of cells. Stable clones isolated from transfections by pGEmin-Gal4 and pGE2min-Gal4 showed that most cells express low levels of reporter genes, as expected from silenced transgenes. Furthermore, GalVP16 transfection in these clones confirmed that DsRed and GFP over-expression can be also detected when integrated, in spite of this silencing, meaning that gene expression resulting from the balance between promoter force and telomeric silencing is correctly equilibrated. Then, the silenced clones described in Fig.12 A appear as ideal models to assess the boundary activity of CTF1 derivatives and to establish experiments that would address to the previously mentioned objectives.

III. TRANSCRIPTION FACTOR CTF1 ACTS AS A CHROMATIN DOMAIN BOUNDARY THAT SHIELDS HUMAN TELOMERIC GENES FROM SILENCING.

This chapter is based on a manuscript currently in revision for publication in Molecular Cell Biology (MCB): Germain Esnault, Danielle Martinet, Jacques S. Beckmann, and Nicolas Mermod. Transcription factor CTF1 acts as a chromatin domain boundary that shields human telomeric genes from silencing. All the figures were generated by the author of this report, except for the FISH experiments.

III.1 Abstract.

Telomeres are associated with chromatin-mediated silencing of genes in their vicinity. However, how epigenetic marks mediate mammalian telomeric silencing and whether specific proteins may counteract this effect is not known. We evaluated the ability of CTF1, a DNA- and histone-binding transcription factor, to prevent the silencing of genes inserted at human cell telomeres. CTF1 was found to protect the gene from silencing when its DNA-binding sites are interposed between the gene and the telomeric extremity, while it does not affect a gene adjacent to the telomere. Protein fusions containing CTF1 histone-binding domain displayed similar activities, while mutants impaired in their ability to interact with the histone did not. Chromatin immuno-precipitation indicated that the propagation of a hypoacetylated histone structure was dependent on the telomere. The CTF1 fusion protein was found to recruit the H2A.Z histone variant at the telomeric locus and to restore high histone acetylation levels to the insulated telomeric transgene. Interestingly, levels of trimethylated H3K9, H4K20 and H3K27 were also increased on the insulated transgene, indicating that these marks may mediate expression rather than silencing at human telomeres. Overall, these results indicate that transcription factors can act to delimit chromatin domain boundaries at mammalian telomeres, thereby blocking the propagation of a silent chromatin structure.

III.2 Introduction.

In eukaryotes, gene expression regulation is believed to rely largely on modifications of the structural organization of chromatin, which may include the relative positioning of chromosomal domains in the cell nucleus, nucleosome localization on regulatory sequences, as well as covalent modifications of histones and DNA or the incorporation of histone variants. For instance, the heterochromatin structure frequently associated with gene silencing has been associated with low levels of histone acetylation and with a variety of other epigenetic marks such as changes in the methylation status of histones and of the DNA. Silent heterochromatic portions of the chromatin are interspersed with euchromatic structures that are more permissive for gene expression, and boundaries between the two types of chromatin structures has been found to be enriched with specific epigenetic markers, such as incorporation of the H2A.Z histone variant (Li et al., 2005; Meneghini et al., 2003; Zhang et al., 2005).

Constitutive heterochromatin, as found at telomeres or centromeres, has been associated with the silencing of adjacent genes. In *S. cerevisiae*, the telomere position effect (TPE) has been well studied and it is attributed to the spread of the SIR complex from the telomere along the chromosome. Proteins of the SIR complex associate with deacetylated nucleosomes, where their histone-deacetylase activity may modify adjacent histones, allowing auto-propagation of the hypoacetylated structure along the chromosome (Hoppe et al., 2002). TPE-associated gene silencing has also been observed in human and mouse cells (Baur et al., 2001; Pedram et al., 2006), but a potential role of SIR-like proteins and/or of another mechanism that propagates a telomeric heterochromatic structure remains to be identified in mammals.

The mammalian telosome is composed of a multiprotein complex that binds to repetitive telomeric DNA sequences. This complex, named Shelterin, may shield the telomere from being recognized as a double-strand break through the formation of the T-loop (Blasco, 2007; de Lange, 2005; Hockemeyer et al., 2006), while insuring the maintenance of a correct length of telomeric repeats by interacting with the telomerase (Kelleher et al., 2005; Ye et al., 2004). Chromatin features present at telomeres and nearby subtelomeric sequences include histone modifications such as the trimethylation of H3 on lysine 9 (H3K9Me3) and of H4 on lysine 20 (H4K20Me3, ref. (Benetti et al., 2007a; Peters et al., 2001; Schotta et al., 2004)). These modifications have been associated with constitutive heterochromatin, as exemplified by the interaction of H3K9Me3 with Heterochromatin Protein 1 (HP1, ref. (Cowell et al., 2002;

Perrini et al., 2004; Schotta et al., 2004)), although other studies have indicated that H3K9Me3 and H4K20Me3 modifications may also occur transiently upon transcriptional activation (Vakoc et al., 2005). Trimethylation of H3K9 and H4K20 are catalyzed by the Suv39H1/2 and Suv4-20H1/2 histone methyl-transferases that are specifically recruited to telomere (Benetti et al., 2007a; Garcia-Cao et al., 2004; Kourmouli et al., 2004). The relatively low activities of the cellular demethylases capable of removing methylation residues at the telomeric locus is consistent with the persistence of these modifications in the constitutive heterochromatin (Cloos et al., 2006; Klose et al., 2006; Loh et al., 2007; Tsukada and Zhang, 2006).

Insulators and boundaries are DNA elements that may alter gene expression by preventing activation or inhibitory effects that stem from their chromosomal environment (Bell et al., 2001; West et al., 2002). Insulators are often defined as DNA elements that can prevent the action of an enhancer or silencer on a promoter, when interposed between the promoter and the regulatory sequence. Chromatin domain boundaries are defined as elements that prevent the propagation of chromatin features such as heterochromatin, and they may thereby demarcate chromosomal domains that possess distinct chromatin features and gene expression status. Nonetheless, while insulator and boundary elements may be distinguished experimentally, the frontier between these two types of epigenetic regulators is not entirely clear, as expected from the fact that enhancers or silencers' function may include the regulation of chromatin structure. For instance, the chicken HS4 enhancer-blocking insulator has also been found to protect transgenes from TPE (Rincon-Arano et al., 2007). In yeast, boundary activities have been observed for proteins such as DNA-binding transcription factors (Fourel et al., 2001; Ishii et al., 2002). Notably, the activation domain of the human transcription factors such as CTF1 have been found to block the SIR-mediated silencing when recruited to the yeast telomere (Ferrari et al., 2004). However, mammalian and yeast cells have distinct chromatin structures, and whether silent chromatin may propagate from mammalian telomeres as it does in yeast is unclear, because an equivalent of the yeast SIR protein complex has not been found in mammalian cells. In addition, whether DNA-binding proteins such as transcription factors may insulate genes from telomeric silencing effects remains to be established.

In this study, we have evaluated whether proteins such as CTF1 may prevent the silencing of telomeric transgenes in mammalian cells, and whether this may result from the interruption of the propagation of a specific chromatin structure from the telomere. To do so, we generated a

dual reporter gene assay, where gene expression of a telomere-distal and of a telomere-proximal locus, separated by a potential insulator sequence, can be assessed simultaneously in individual cells. We found that the viral VP16 transcriptional activation domain activates both the telomere proximal and distal genes. In contrast, native CTF1, or fusion protein containing its histone-binding domain, were found to prevent the silencing of the telomere-distal transgene only. Chromatin immuno-precipitation experiment (ChIP) indicated that CTF1 can demarcate chromatin structures of distinct histone acetylation status and to recruit H2A.Z at the chromatin domain boundary. Furthermore, the H3K9Me3 modification was found on insulated telomeric transgenes, thus marking gene expression at an otherwise silent chromatin locus. Overall, we conclude that transcription factors such as CTF1 may mediate chromatin domain boundaries to protect transgenes from the propagation of a silent telomeric chromatin structure.

III.3 Material and methods.

III.3.1. Plasmid vectors.

The minimal CMV promoter and EGFP and DsRed coding sequences (Clontech) were PCR amplified and cloned in both orientations in pBS-SK2 containing telomeric repeats, kindly provided by J. Baur (Baur et al., 2001). Puromycin resistance gene expressed from the CAG promoter was inserted upstream of DsRed, in a telomere-distal position. Four Gal4 binding site were introduced between EGFP and DsRed expression cassettes at AscI and BamHI restriction sites, yielding pGE1min-Gal and pGE2min-Gal. Control plasmids were generated by deletion of the telomeric repeats. Plasmids encoding the Gal DNA binding domain alone (pCD-Gal-DBD), or fused to the CTF1 Proline rich (pCMV-Gal-Pro) or to the VP16 (pCMV-Gal-VP16) transcriptional activation domains were as described previously (Pankiewicz et al., 2005). Plasmids encoding Gal-CTF1 fusion mutations were previously described by Alevizopoulos *et al*. (1995). Plasmids used to generate stable populations expressing Gal4 derivatives were obtained by cloning Gal-fusion genes or the BFP gene in an expression vector carrying the MAR 1-68 and the SV40 promoter (Girod et al., 2007) .

III.3.2. Cells culture, transfection and *in situ* hybridization.

HeLa cells (Clontech) were cultivated at 37°C and 5% CO2 in DMEM-F12 with 10% fetal bovine serum (Gibco). Histone deacetylation or DNA methylation studies were performed by supplementing the cell culture medium with either 1µM of Trichostatin A (TSA, Wako) for 48h, 1mM of Sodium butyrate (Sigma) for one week, 3µM of 5-aza-2'-deoxycytidine (5azadC, Sigma) for 48h, or 50µM of Bromo-deoxyuridine (BrdU, Applichem) for one week. Transfections were performed using the Fugene 6 transfection reagent following instructions from the manufacturer (Roche). Stable clones were obtained by transfection of linearized plasmids pGEmin-Gal, pGE2min-Gal or their respective controls. Cells were selected with 2µg/mL of puromycin for three weeks, and all analyses were performed at least two weeks after the end of selection, to allow for the silencing of the telomeric locus. Transient transfections were performed by co-transfection of a Gal-fusion encoding plasmid and a BFP encoding plasmid at a molar ratio of 9:1. Cytofluorometric assays of the fluorescent reporter proteins were performed 48h later. Stable populations expressing Gal-DBD/Gal-Pro were obtained by co-transfecting the Gal-fusion expression plasmid, a BFP-encoding plasmid, and a Zeocin resistance plasmid at 45:45:10 weight ratio.

Zeocin resistant cells displaying high BFP levels were sorted twice, and the amount of zeocin was increased to 1800µg/mL with increments of 200µg/mL, to insure consistent and elevated levels of fusion protein expression. Fluorescence *in situ* hybridization (FISH) was performed as described previously (Flahaut et al., 2006; Girod et al., 2007) using two colors labeling of the reporter plasmids and of the telomeric repeats.

III.3.3 Chromatin immuno-precipitation.

Antibodies against acetylated H3 (06-599), acetylated H4 (06-866) and trimethylated H3K9 (07-442) were obtained from Upstate biotechnology. Antibody against H2A.Z (ab4174) was purchased from Abcam. HeLa cells were harvested at a confluence of 90% and cross-linked with 1% formaldehyde for 4 min. After lysis of the nuclei, chromatin was sonicated to obtain fragments of ~1000pb and digested with BamHI. The chromatin solution was diluted to a volume of 300µL in a buffer containing 200mM HEPES, 2M NaCl, 20mM EDTA, 0.1% NaDoc, 1% Triton X-100, 1mg/mL BSA. Chromatin fragments were precleared 30 min with 10µL rProtein A Sepharose (Amersham Biosciences) and supernatants were incubated at 4°C overnight with 5µL of antibody. Immunoprecipitated complexes were incubated with 10µL rProtein A Sepharose and pellets were washed 3 times with IP buffer (20mM HEPES, 0.2M NaCl, 2mM EDTA, 0.1% NaDoc, 1% Triton X-100). Immunoprecipitated complexes were eluted in 100mM Tris/HCl, 1% SDS and cross-links were reversed at 65°C for 1 hour. Precipitated DNAs were eluted in 50µL TE.

III.3.4. Quantitative PCR.

Quantitative PCR was performed on 7700 Sequence detector (Applied Biosystems) using SYBR Green reagent (Eurogentec). Chromatin immuno-precipitation samples and chromatin input were diluted 10 fold before analysis. GAPDH amplification was performed using 5'-CGCCCCCGGTTTCTATAA-3' and 5'-ACTGTCGAACAGGAGGAGCAG-3' primers, EGFP using 5'-AGCAAAGACCCCACCGAGAA-3' and 5'GGCGGCGGTCACGAA-3' primers and DsRed using 5'-TTCCAGTACGGGTCCAAGGT-3' and 5'-GGAAGGACAGCTTCTTGTAGTCG-3' primers. EGFP and DsRed values were normalized by GAPDH.

III.4. Results.

III.4.1. Design of a quantitative assay of telomeric gene silencing.

In order to analyze both telomere-insulated and non-insulated genes co-integrated at the same telomeric locus, we generated the reporter plasmids shown in Fig. 13A. Reporter vectors consist of the green fluorescent protein (GFP) and red fluorescent protein (DsRed) coding sequences placed on either side of four DNA binding sites for the yeast GAL4 protein. Each reporter gene was placed under the control of a minimal CMV promoter, in an orientation mediating either convergent sor divergent directions of transcription. An antibiotic resistance gene was placed adjacent to DsRed, while telomeric (TTAGGG)n repeats were placed next to the GFP expression cassette. Previous studies have demonstrated that stable transfections of telomeric repeat-containing plasmids yield mostly single copy integration at a telomeric position, possibly because integration of the telomeric repeats induces a chromosomal break and the formation of a new telomere (Baur et al., 2001; Pedram et al., 2006; Rincon-Arano et al., 2007). These constructs were transfected, and antibiotic-resistant cells having stably integrated the transgenes in their genome were selected and sorted into monoclonal populations. Clones showing the following properties were discarded: (1) heterogeneous or disproportionate DsRed and GFP fluorescence, probably because of multiple insertions and/or a non-clonal nature, (2) no activation of DsRed and/or GFP upon transfection of a Gal-VP16 expression vector, which suggests the deletion of one or both reporter genes, and (3) high basal expression of GFP and DsRed, which may result from non-telomeric integrations. Fluorescence *in situ* hybridization (FISH) analysis indicated a telomeric or subtelomeric transgene position for all retained clones (see Fig. S1 in the supplemental material). Monoclonal populations were also generated from the transfection of reporter plamids deleted of the telomeric repeats, to obtain integration at non-telomeric loci, and cell clones were selected similarly according to the above criteria 1 and 2.

This yielded three categories of clonal populations. The first two categories, generated from telomeric repeat-containing plasmids, display a telomeric or subtelomeric transgene location and nearly undetectable reporter gene expression, or low but detectable transgene expression (see Fig. S1 in the supplemental material and Fig. 12A and 12B). These results are consistent with previous reports of the low expression of telomeric transgenes in mammalian cells (Baur et al., 2001; Pedram et al., 2006; Rincon-Arano et al., 2007). The last category of clones generated using constructs devoid of telomeric repeats, displayed random

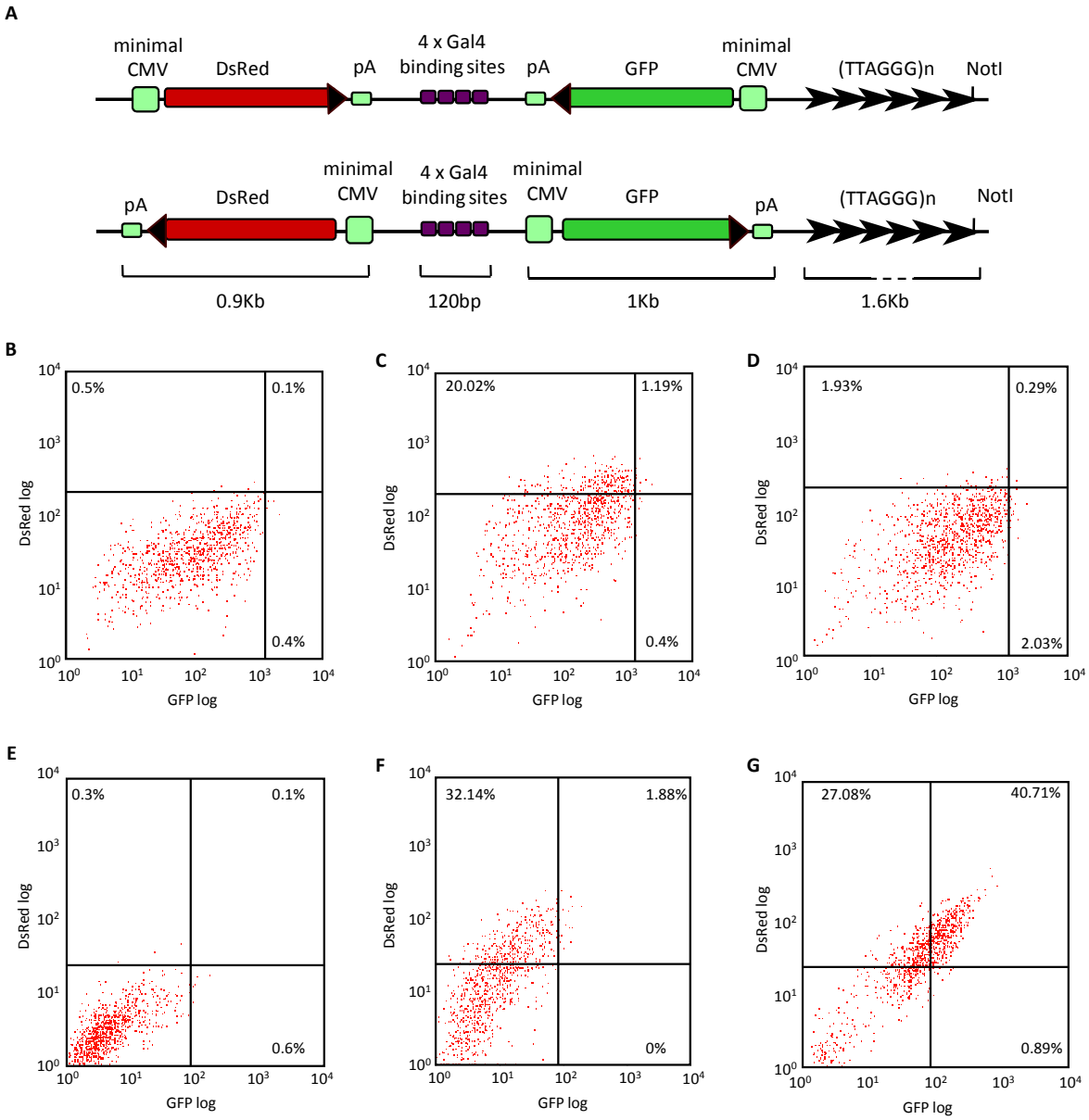


Fig 13. Gal-Pro protects transgenes from telomeric position silencing effects.
(A) Vectors used to assay the silencing of telomeric transgenes. Constructions were designed to place the GFP-coding gene in a telomere-proximal position and DsRed in a telomere-distal position relative to four binding sites for the yeast GAL4 protein. Transcription of the reporter genes is either convergent or divergent. Not depicted here is an antibiotic selection gene located to the left of the DsRed gene. Control plasmids used for random integration at internal chromosomal locations were deleted of the telomeric repeats which are shown by arrowheads. (B-G) Examples of cytofluorometric analysis of the DsRed and GFP fluorescence in clones B09 (panels B, C, D) and D17 (panels E, F, G), carrying at a telomeric position shown in panel A the convergent or divergent reporter construct, respectively. Each clone was transiently co-transfected with a plasmid encoding the GAL4 DNA-binding domain alone (Gal-DBD, panel B and E) or fused to the CTF1 proline-rich (Gal-Pro; C, F) or VP16 (Gal-VP16; D, G) activation domain, and with a BFP expression vector. The panels depict the GFP and DsRed fluorescence of 1000 BFP-expressing cells. Quadrant regions were set for each clone according to the basal DsRed and GFP fluorescence in Gal-DBD expressing cells, so as to obtain 99% of the cells in the bottom left region. The percentile of cells in each quadrant is indicated.

chromosomal integration sites and variable levels of expression (see Fig. 12C and S2 in the supplemental material). Clones displaying clear internal chromosome integration and relatively low expression levels were kept as controls.

III.4.2. CTF1 protects telomeric transgenes from TPE.

The proline-rich domain of CTF1 has been shown to interact with histone H3.3 and to activate gene transcription in response to growth factors in mammalian cells (Alevizopoulos et al., 1995). To specifically assess CTF-1 activity at mammalian telomeres, and to exclude possible interference from other members of the HeLa cell CTF/NF1 family (Santoro et al., 1988), the CTF1 proline-rich domain was transiently expressed as a fusion to the DNA binding domain of the yeast GAL4 protein (Gal-Pro). Expression vectors encoding either the unfused GAL4 DNA-binding domain (Gal-DBD), or a fusion with the strong herpes simplex virus VP16 activator (Gal-VP16), were used as controls. These plasmids were co-transfected with a blue fluorescent protein (BFP) expression vector as a transfection marker, and transiently transfected BFP expressing cells were analyzed for GFP and DsRed fluorescence. Gal-Pro expression resulted in an increase of DsRed fluorescence without an increase of GFP fluorescence in the telomeric clones (compare Fig. 13B and 13E with 13C and 13F, respectively), while the Gal-VP16 fusion did not significantly activate the transgenes, when transcribed in a convergent fashion (compare Fig. 13B and 13D), or it activated DsRed and GFP divergent transcription to a similar extent (Fig. 13E and 13G). The low activation seen in Fig. 13D is explained by the more distant location of the Gal-VP16 binding sites from the promoters driving the transcription of the convergent reporter genes (Figure 13A, top drawing). Assays of GAL4 fusions to other proteins that bind insulator and/or boundary elements, such as CTCF or USF1 (Baur et al., 2001; Pedram et al., 2006; Rincon-Arano et al., 2007), failed to affect DsRed or GFP expression (data not shown), confirming a specific function of CTF1 at the telomeric loci.

Quantification of the Gal-Pro effect indicated that it occurs in independent clones that have a telomeric transgenes integrated in various chromosomes (Fig. 14A and 14B, and Fig. S2 in the supplemental material). In contrast, Gal-VP16 activated the expression of the reporter genes to a variable extent, but without a marked preference for the activation of DsRed over GFP. Gal-Pro had variable but generally smaller effects on the expression of transgenes integrated at non-telomeric positions, where it could also activate GFP expression (Fig. 14C).

We previously showed that the proline-rich activation domain of CTF1 possesses two regions that cooperate to bind histone H3, and that this domain may reposition nucleosomes close to its binding site (Ferrari et al., 2004; Muller and Mermod, 2000; Pankiewicz et al., 2005). Thus, we assessed whether the H3 interaction domains may mediate the boundary activity. Gal-fusions previously characterized by their ability to bind H3 were expressed in telomeric clones B09 and D17, where Gal-Pro shows strong boundary effects. In both cases, deletion of the H3 interaction domains was associated with a strong reduction of DsRed expression (see Fig. S3 in the supplemental material). Single proline rich domain point mutations known to decrease or abolish interaction with H3 similarly decreased the boundary effect (see Fig. S4 in the supplemental material), supporting a role for the H3 interaction in the boundary activity.

To assess if the boundary effect can also be observed from the expression of native transcription factors such as CTF1, we analyzed clones generated with reporter constructs carrying seven CTF/NF1 binding sites inserted between the two reporter genes instead of the GAL4 sites. Since various members of the family of CTF/NF1 proteins are expressed in HeLa cells (Santoro et al., 1988), we sought to identify clones in which the additional expression of CTF1 may mediate a boundary effect. Clones having integrated the reporter genes in telomeric or internal chromosomal positions were thus isolated and analyzed after the transient expression of CTF1. The boundary effect was observed upon CTF1 expression in cells with telomeric transgenes (Fig. 15A and 15B), while a commensurate activation occurred for both reporter genes inserted at an internal location on the chromosome (Fig. 15C and 15D). The boundary effect at telomeric loci was observed in three independent clones with telomeric transgenes, but the boundary effect observed upon CTF1 over-expression was overall smaller that that obtained with the GAL4 fusion protein (data not shown). This may

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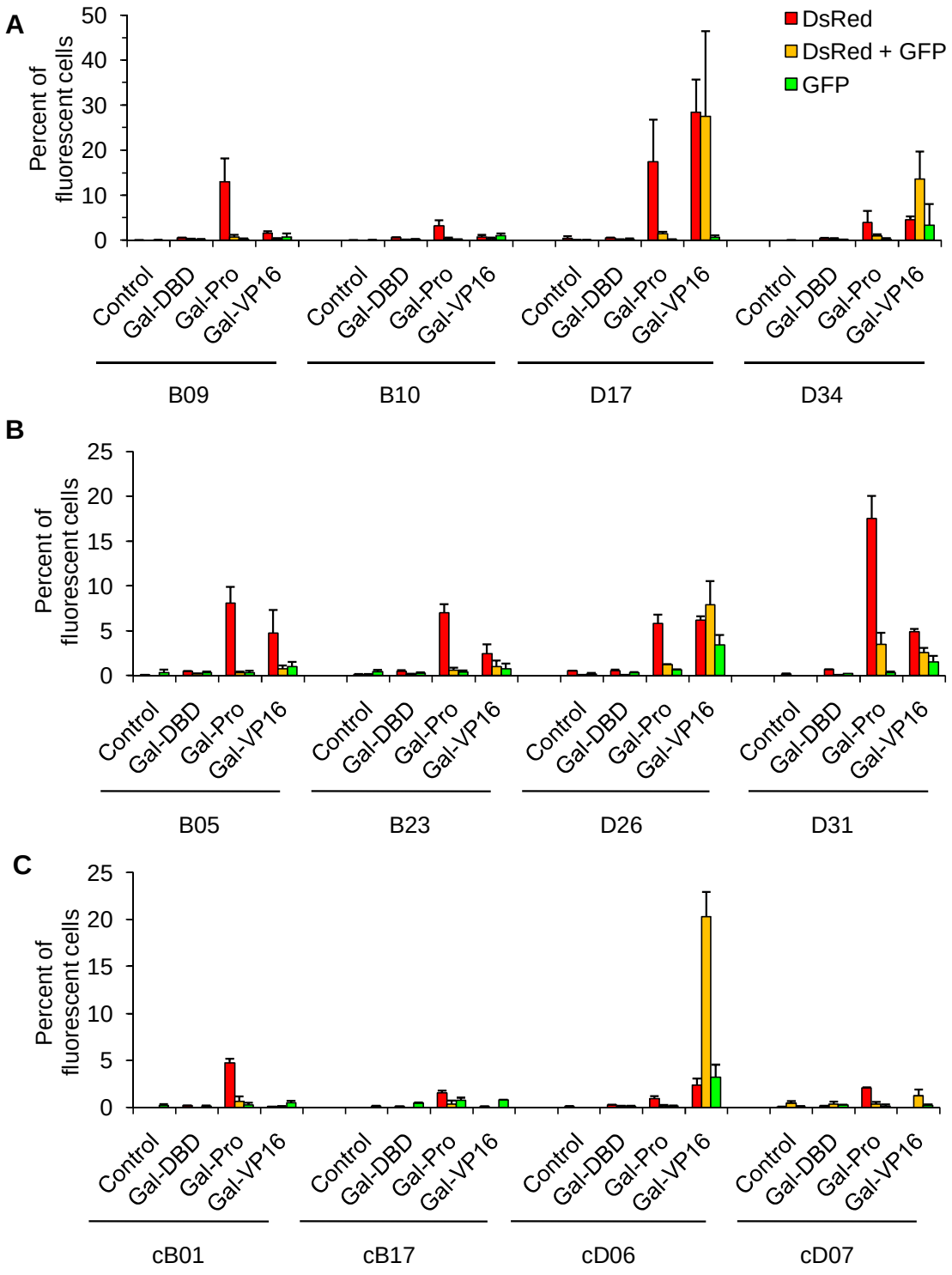
Figure 14. Specific boundary activity of Gal-Pro at telomeric transgenes.
(A, B) Panel A depicts the percentile of fluorescing cells from cell clones having integrated a reporter construct at a telomeric locus, and which display little or no basal expression of the transgenes, whereas panel B shows results from clones generated similarly, but having detectable levels of GFP or DsRed fluorescence. B-labeled and D-labeled clones were generated using the convergent or divergent, respectively, reporter constructs containing telomeric repeats. Each clone was transiently transfected with an empty expression vector (control), or with the Gal-DBD, Gal-Pro or Gal-VP16 expression vector. Values represent the average of the percentile of cells expressing DsRed, GFP, or high levels of both DsRed and GFP among 1000 BFP-expressing cells, determined as illustrated in Figure 13. Error bars: standard error of the mean of at least 3 independent experiments. (C) Clones generated without telomeric repeats, and showing internal integration site, were transfected and processed as for panels A and B.


A
Percent of fluorescent cells
50
40
30
20
10
0
DsRed
DsRed + GFP
GFP
Control
Gal-DBD
Gal-Pro
Gal-VP16
B09
B10
D17
D34
B
Percent of fluorescent cells
25
20
15
10
5
0
B05
B23
D26
D31
C
Percent of fluorescent cells
cB01
cB17
cD06
cD07

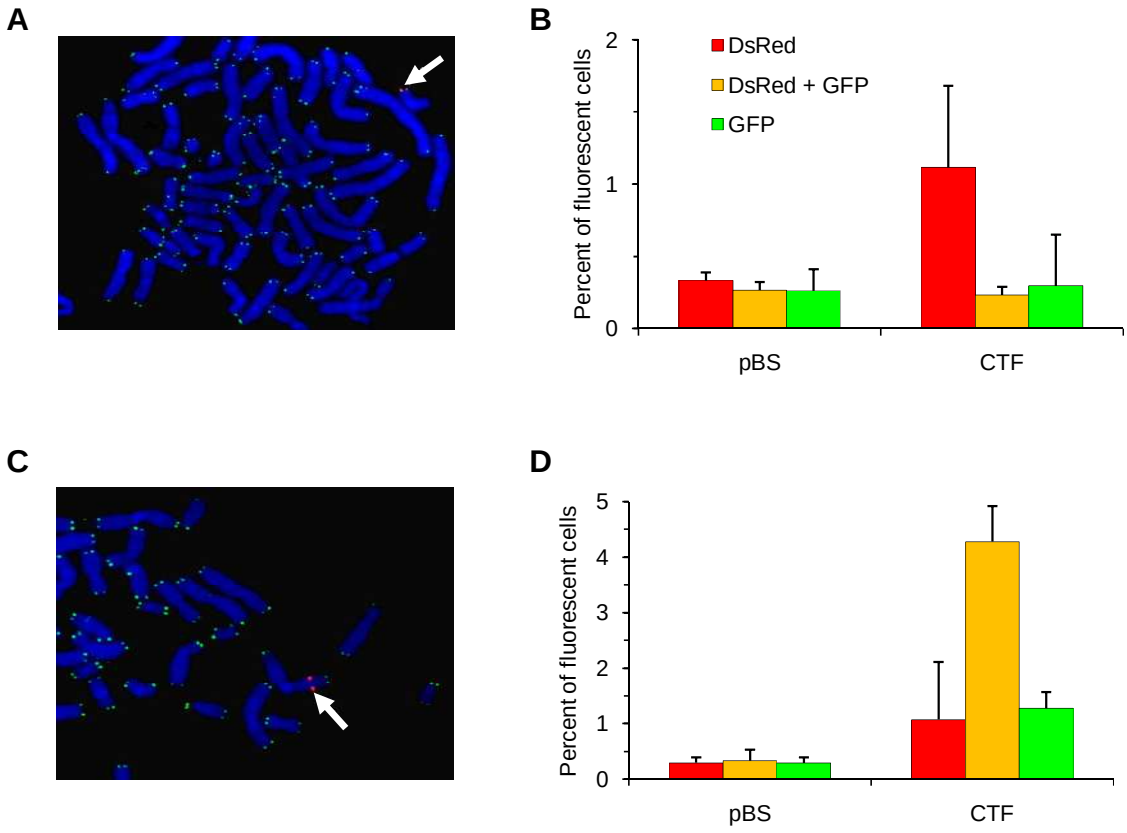


Figure 15. Native CTF1 acts as boundary at human cell telomeres.
HeLa cells were transfected with a plasmid reporter construct as in Figure 1A, except that it carries seven CTF/NF1 binding sites instead of GAL4 sites inserted between the DsRed and GFP genes, which are divergently transcribed. The integration site of clones generated with reporter constructs containing (panels A and B) or devoid of (panels C and D) telomeric repeats was verified by FISH analysis by probing telomeric repeats (green) or the reporter vector (red) (panels A and C). Boundary activity was evaluated by comparing DsRed and GFP expression as described Figure 2 in clones transiently co-transfected with a control plasmid (pBS) or with the CTF1-encoding expression plasmid (CTF) (panels B and D).

stem from the background of CTF/NF1 proteins, as they may already mediate some boundary effects on the reporter constructs containing CTF/NF1 binding sites, and/or from the stronger interaction of GAL4 to heterochromatic DNA as compared to CTF1 (Pankiewicz et al., 2005).

Taken together, these results indicate that CTF-1 and its fusion derivatives act specifically to prevent silencing of the telomere distal but not of the telomere-proximal gene, implying that they may prevent the propagation of a silencing signal from the telomere towards more centromeric sequences. Thus, these results suggested that this protein may act as a boundary or barrier element that blocks the spread of a repressive chromatin structure from the telomere.

III.4.3. Chromatin landscape at mammalian telomeric *loci*.

Chemical agents that affect histone acetylation or DNA methylation were used to assess whether telomeric transgenes are subjected to chromatin-mediated silencing effects. Trichostatine A (TSA), a broad-specificity inhibitor of class I and II histone deacetylase (HDAC) was found to strongly increase transgene expression at various telomeric positions in independent cell lines (see Fig. S5 in the supplemental material). In contrast, sodium butyrate (NaB), a more specific inhibitor of HDAC I and IIa classes, mediated lower unsilencing effects in some clones, suggesting an involvement of the HDAC IIb class in gene silencing at some but not all telomeres. Thus, several HDAC activities may be involved in telomeric gene silencing. HDAC inhibitor treatment of telomeric clones with lower transgene expression generally resulted in greater enhancement of gene expression, as would be expected from a chromatin-mediated silencing process (compare Fig. S1A and S1B with S5A and S5B in the supplemental material).

Treatment of telomeric clones with the 5-aza-2’-deoxycytidine (5azadC) DNA-methylation inhibitor had little effect on transgene expression (Figure S6). Thus, DNA methylation is unlikely to be the primary determinant of telomeric silencing in this cellular model. Several studies have shown that Bromodeoxyuridine (BrdU) can abolish expression variegation, namely the cycling between semi-stable expressing and non-expressing states. Its mode of action remains unclear, but it may act by decreasing histones mobility (Lin et al., 1976). BrdU treatment of telomeric clones was associated with an increase in expression of the reporter genes, but to a lesser extent than that noted with TSA, suggesting that telomeric silencing involves chromatin remodeling.

The involvement of nucleosome hypoacetylation in the silencing of telomeric genes was further analyzed by chromatin immunoprecipitation assays (ChIP) of two clonal populations showing strong telomeric silencing. This revealed hypoacetylation of H3 over both the GFP and DsRed telomeric sequences, but the effect was more prominent on the telomere-proximal GFP gene, as compared to the telomere distal DsRed sequence. This does not stem from preferential acetylation of the latter gene, as high levels of acetylated H3 were found on both transgenes integrated at an internal locus in the cD06 cells (Fig. 16A and 16B). Hypoacetylation of histone H4 was only observed on the GFP sequence, further arguing for a correlation between telomere proximity and the histone hypoacetylation effect (Fig. 16B). This finding is consistent with the spread of a silencing signal from the telomeric repeats, and it is reminiscent of distance-related silencing effects associated with the propagation of a silent chromatin structure from yeast telomeres (Kurdistani and Grunstein, 2003).

The trimethylation of histone H3 on lysine 9 (H3K9Me3) has been associated with heterochromatin-mediated gene silencing (Benetti et al., 2007a; Peters et al., 2001; Schotta et al., 2004). However, H3K9Me3 levels were not significantly elevated in the telomeric clones as compared to the transgenes integrated at an internal position (Fig. 16C). Rather, low H3K9Me3 modifications on clone D17 GFP sequence correlates well with the low GFP expression, in contrast to clones B09 and cD06 which show moderate or high levels of both methylation and GFP expression, respectively (compare Fig. 16C and Fig. S1 in the supplemental material). Other histone modifications such as H4K20Me3, H3K27Me3 or H3K79Me2 did not have a preferred location on the telomeric genes (data not shown). The histone variant H2A.Z has often been located at the boundaries of silent and permissive chromatin domains (Dhillon and Kamakaka, 2000; Meneghini et al., 2003). Its low levels at the telomeric reporter genes of clones B09 and D17 indicate that it may be excluded from telomeric loci (Figure 16D). Overall, these results link telomeric gene silencing to histone H3 hypoacetylation and H3K9 methylation, and they imply that a short-ranging gradient of such modifications stems from the telomere.

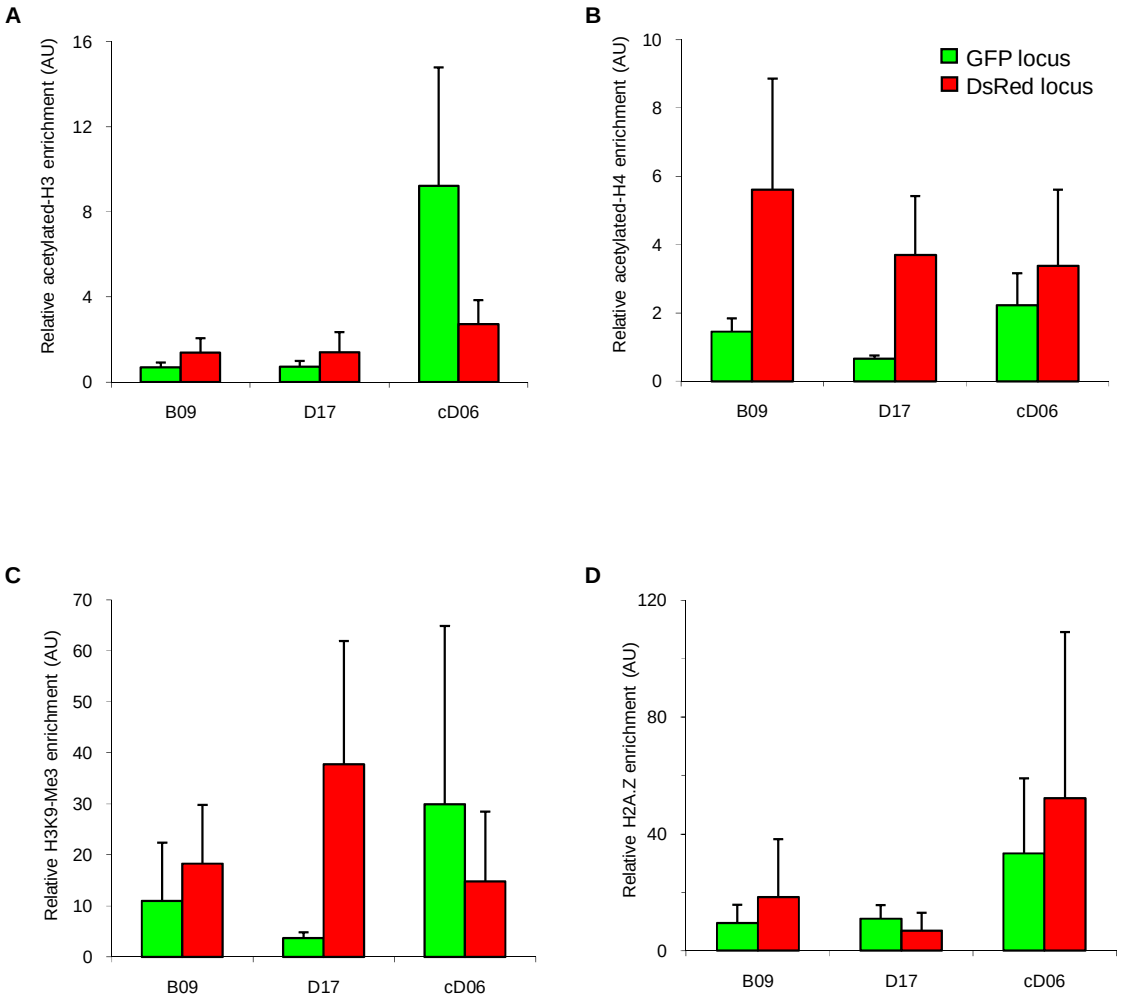


Figure 16. Telomeric histones H3 and H4 are hypoacetylated.
Chromatin immuno-precipitation were performed on two telomeric clones (B09 and D17) and one clone with non-telomeric integration (cD06). Chromatin fragments were immunoprecipitated using antibodies specific for acetylated H3 and H4 (panel A and B), trimethylated H3K9 (C) and the histone variant H2A.Z (D), and the precipitated DsRed and GFP genomic sequences were assayed by real-time PCR, and normalized to values obtained by amplifying the GAPDH gene. Mean and SEM of 3 independent experiments with at least two independent chromatin preparation are indicated.

III.4.4. CTF1 fusion protein delimits distinct chromatin domains at telomeric boundaries.

Given our conclusion that telomeric transgene silencing involves histone modifications, we next assessed if Gal-Pro expression may selectively oppose these changes over the DsRed-coding sequence. Clone B09 was stably transfected with Gal-DBD or Gal-Pro expression vectors to insure stable expression of the GAL4 fusion in a significant proportion of the cell population. Expression of these GAL4 fusions was assessed indirectly, by measuring the fluorescence of the blue fluorescent protein (BFP) expressed from a co-transfected vector.

Gal-pro expression was associated with an increase of H3 and especially H4 acetylation on the DsRed sequence of clone B09. However, Gal-Pro expression did not affect histone acetylation on the GFP sequence, indicating that Gal-Pro mediates the formation of two chromatin domains of distinct acetylation status, but that it does not act by recruiting HATs that would acetylate bidirectionally the GFP and DsRed genes. Gal-Pro expression also strongly increased H3K9Me3 on DsRed but not GFP at the B09 telomere. The trimethylation of H3K27 and H4K20, which are modifications generally associated with gene silencing, were similarly increased on the expressed DsRed sequence in the presence of Gal-Pro (data not shown). The HDAC inhibitor TSA yielded an increase of the acetylation of both DsRed and GFP, as well as the trimethylation of H3K9, indicating that the latter modification may be a consequence of the increase in histone acetylation.

To determine if histone acetylation changes are always involved in the boundary effect, clone D17 was similarly tested, as GAL-Pro has strong boundary activity while the HDAC inhibitor NaB has little effect on telomeric gene expression (Fig. 14A and Fig. S5 in the supplemental material). Expression of Gal-Pro was not associated with an increase in H3 and H4 acetylation, nor with modifications such as H3K9Me3, H3K27Me3 or H4K20Me3 (Fig. 17 and data not shown). However, the occurrence of H2A.Z on DsRed was significantly increased. This indicates that several types of chromatin structures may be associated with telomeric silencing and insulation effects, and that Gal-Pro may act to separate chromosomal domains of distinct chromatin structures.

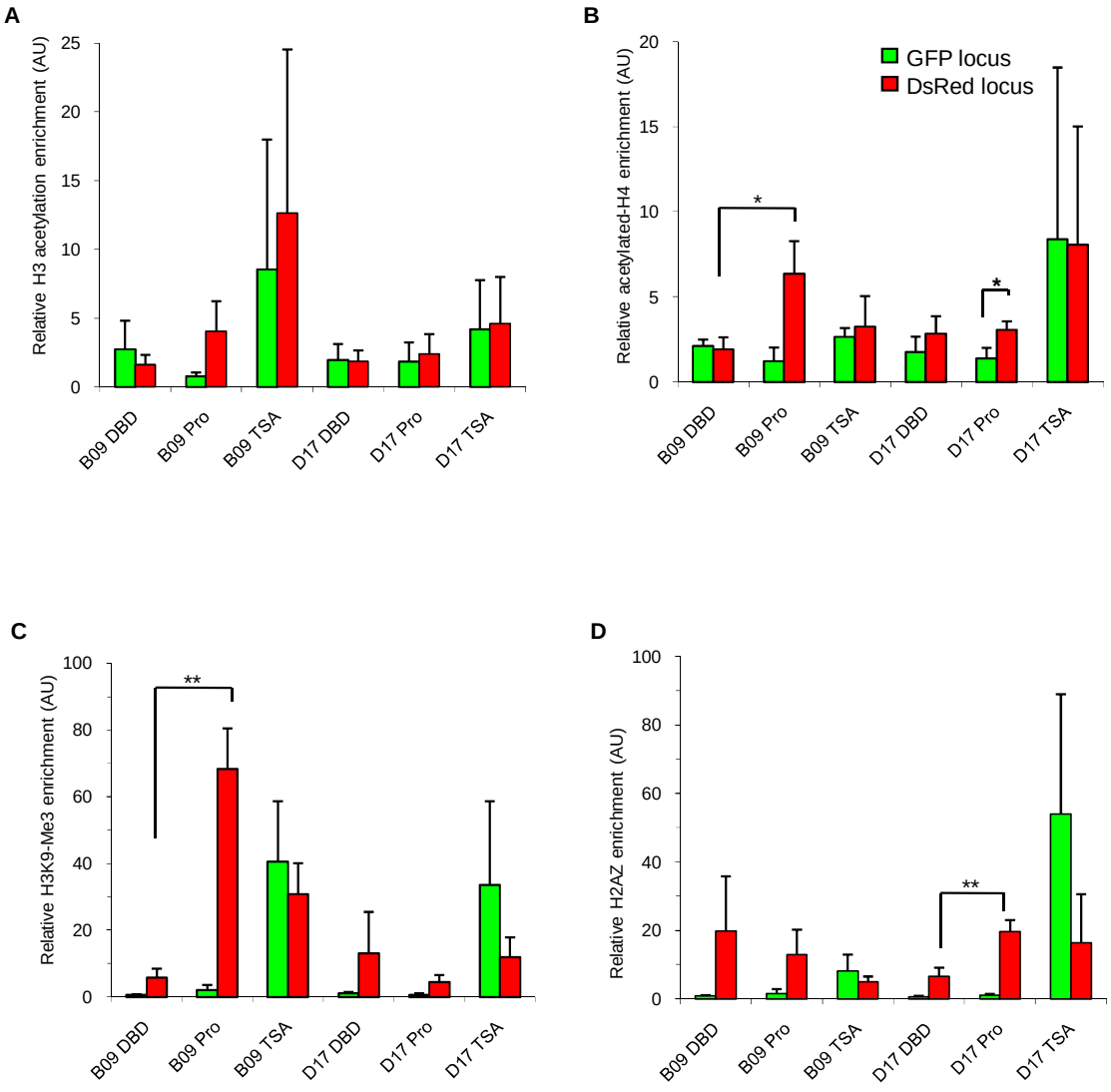


Figure 17. Effect of the Gal-Pro boundary on telomeric chromatin structure.
Chromatin immuno-precipitations were performed on telomeric clones B09 and D17 stably expressing Gal-DBD or Gal-Pro, or treated with the HDAC inhibitor TSA. Antibodies were specific for acetylated H3 and H4 (panel A and B), trimethylated H3K9 (C) and histone variant H2A.Z (D) and precipitated sequences were processed as for Fig. 16. (*) $p<0.05$ Student's *t*-test (**) $p<0.01$ Student's *t*-test.

III.5. Discussion.

The eukaryotic genome is thought to be partitioned in euchromatic or heterochromatic domains in which chromatin may be either permissive for gene expression or rather silent. How the boundaries separating these chromatin domains are established, and how they may influence gene expression, remains poorly understood. In this work, we show that two genes co-localized at a telomeric locus can be partitioned into active and inactive chromatin structures by the CTF1 protein or fusions derived thereof. This mode of action is distinct from that of the VP16 transcriptional activator, which induces bi-directionally the expression of telomere proximal as well as telomere distal genes, but only over a short distance. This latter effect most likely results from the ability of VP16 to recruit HAT and components of the basal transcription machinery to the promoter (Ito et al., 2000). In contrast, CTF1 derivatives protect the telomere-distal gene from silencing effects without significantly affecting the expression of the telomere proximal gene, and irrespective of the gene orientation or distance to the promoter. This implies that CTF1 does not act as a classical transcriptional activator, but rather that it mediates the establishment of a barrier that blocks the propagation of a silent chromatin structure from the telomere, thereby forming a boundary between expressed and silent genes. The CTF-1 boundary effect is mediated by its histone-binding domain, and mutations that inhibit interactions with the histone also inhibit the boundary effect. Taken together with previous observations that CTF1 binds preferentially to the H3.3 and that this histone variant is enriched at chromatin boundaries (Ferrari et al., 2004; Mito et al., 2007), these findings imply a mechanism whereby the interaction of CTF1 with nucleosomes may establish a chromosomal structure that blocks the auto-propagation of silencing signals from the telomere. These findings provide a mechanistic explanation for the previous observations that CTF1 may contribute to reversing chromatin-mediated gene silencing, but that alone it is unable to activate transcription (Pankiewicz et al., 2005).

In budding yeast, TPE is mediated by the SIR protein complex spreading from the telomere over subtelomeric regions, which results in histone deacetylation and gene silencing. However, a similar mechanism involving the propagation of SIR proteins has not been reported in mammalian cells. Rather, the establishment of a repressive telomeric structure has been associated with increased H3K9Me3 modifications at telomeres (Benetti et al., 2007a; Perrini et al., 2004). H3K9Me3 is known to bind HP1, which may in turn recruit the Suv39

HMTase to mediate further H3K9 methylation. Here, we find that histone deacetylation is linked to silencing at several of the analyzed telomeric loci, and that broad-range HDAC inhibitors such as TSA mediate not only an increase of histone acetylation, but also other types of modification such as H3K9 trimethylation. This implies a causal effect of hypoacetylation on histone methylation levels and silencing effects in mammalian cells. This conclusion is further supported by the previous demonstration that H3K9Me3 modifications may occur as a result of gene transcription (Vakoc et al., 2005) and by the occurrence of H3K9Me3 on a transgene protected from a chicken telomere by the cHS4 beta-globin insulator (Pedram et al., 2006; Rincon-Arano et al., 2007).

Although we observed variable degrees of histone hypoacetylation when comparing different telomeric integration loci, the extent of histone deacetylation was found to be associated with telomere proximity, as it is significantly lower over the telomeric-distal gene. This finding suggests a short-ranging spread of a hypoacetylation signal from the telomere. This contrast the long-ranging histone hypoacetylation and silencing that stem from yeast telomeres, and it may explain why telomeric gene silencing has been more difficult to detect in mammalian cells. In human cells, we find that expression of Gal-Pro results in the recovery of histone acetylation on the telomere-distal but not on the proximal gene, further supporting the notion that it acts to block the self-propagation of a deacetylated histone structure. This interpretation is consistent with the recent implication of the mammalian SIRT6 homolog of the yeast Sir2 HDAC in mammalian TPE, and with its H3K9 deacetylase activity (Michishita et al., 2008). Thus, these results suggest a mechanism by which SIRT6 and possibly other proteins may propagate along the mammalian chromosome to silence subtelomeric regions.

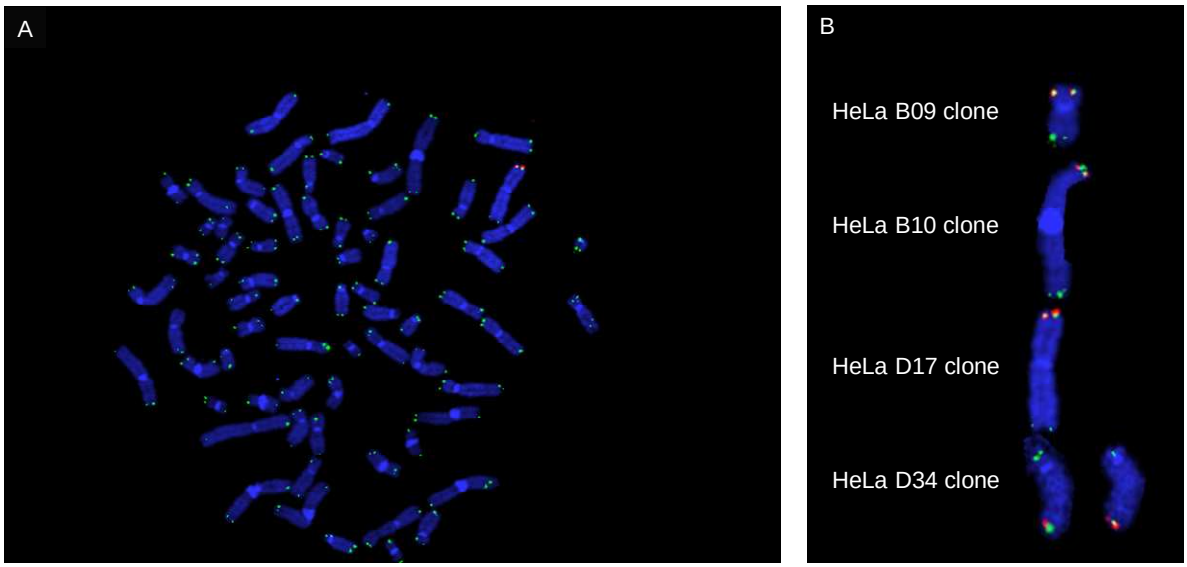
Interestingly, our results imply that various chromatin structures and/or mechanisms may be implicated in the telomeric silencing and boundary effects. For instance, distinct telomeric clones display different responses to treatment with agents that affect chromatin-modifying activities. Furthermore, the boundary effect elicited by the CTF1 fusion protein is not always associated with major changes in histone acetylation, as it was rather associated with the incorporation of the histone H2A.Z variant in the insulated gene of one clone. This finding is reminiscent of the previous demonstration that the yeast H2A.Z homolog is capable of synergizing with boundary elements, and that it preferentially locates on insulated telomeric genes (Li et al., 2005; Meneghini et al., 2003; Zhang et al., 2005). Thus, in contrast to the view that the mammalian H2A.Z may have the distinct function of mediating a silent heterochromatin structure (Fan et al., 2004; Li et al., 2005; Meneghini et al., 2003; Zhang et

al., 2005), our results indicate that it can be associated with gene expression at human telomeres.

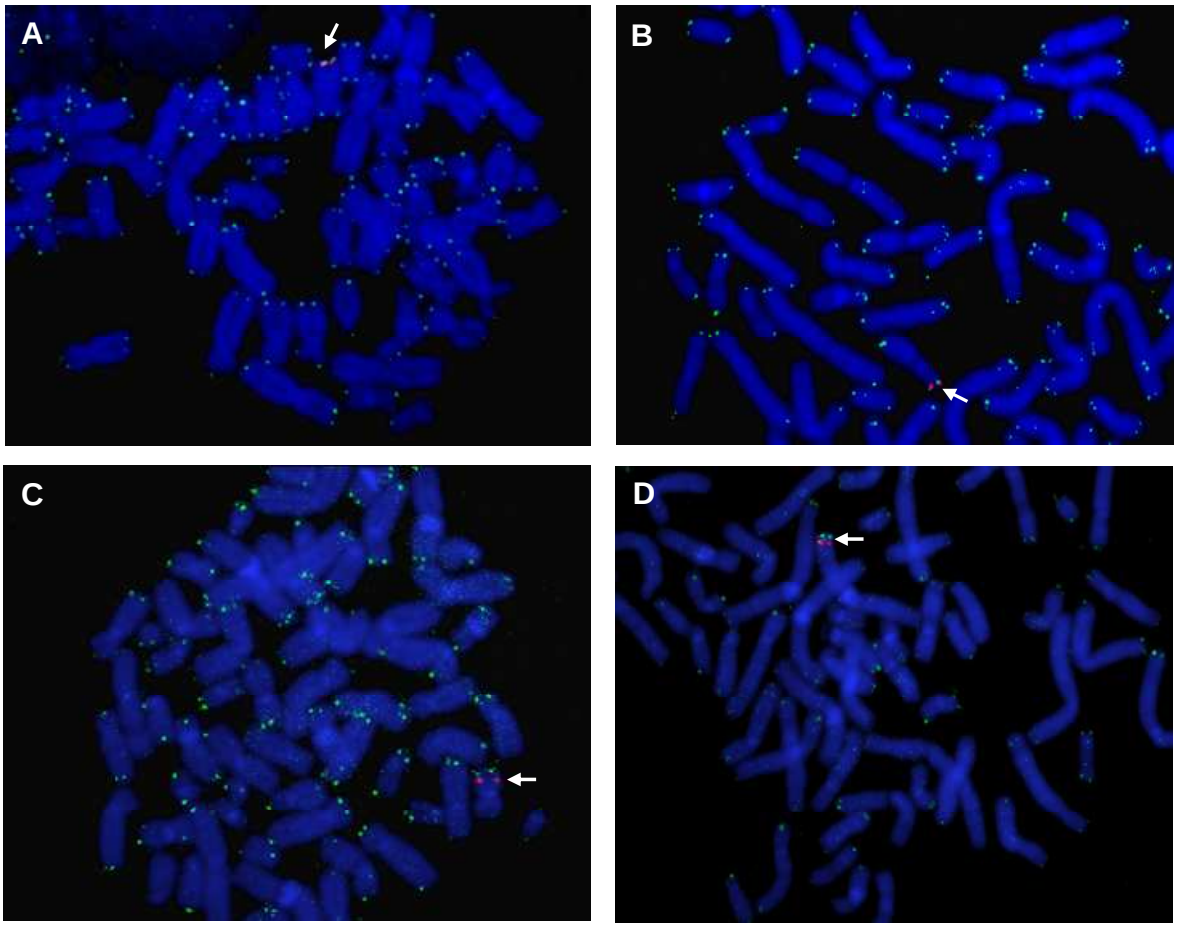
What distinguishes telomeric loci where the boundary effect may be associated with histone acetylation or with H2A.Z enrichment is unclear at present, but it may stem from different chromosomal contexts. It has been found that telomeric silencing is often counteracted by HDAC inhibitors in tumor cell lines but not in normal cells (Baur et al., 2001; Pedram et al., 2006; Rincon-Arano et al., 2007). While our results are consistent with these observations, they raise the possibility that distinct mechanisms may operate at distinct chromosomal loci, and that the previously observed cell-specific behaviors may also reflect distinct telomeric assay systems.

While the role of the CTCF transcription factor as an enhancer-blocking insulator has been well characterized, the occurrence of mammalian DNA-binding proteins that might mediate chromatin-domain boundary effects has remained elusive. For instance, the USF1 transcription factor binding site present in the chicken HS4 insulator has been proposed to mediate the boundary activity of this epigenomic regulator (West et al., 2004). However, while HS4 can shield transgenes from silencing at chicken telomeres, the USF1 protein was found to be dispensable for this effect (Pedram et al., 2006; Rincon-Arano et al., 2007). Thus, evidence for the long sought DNA-binding activities that may mediate telomeric boundaries in higher eucaryotes could not be obtained. Our results indicate that binding sites for a single transcription factor, or the recruitment of its histone-binding domain by a heterologous DNA-binding activity, suffices to mediate a chromatin domain boundary effect and that it acts to shield transgenes from telomeric silencing effects. In addition, our study provides a means by which very short DNA sequence acting as boundaries may be identified and characterized, opening the way to their use to protect transgenes from silencing effects, for instance by their incorporation in viral or non-viral gene therapy vectors.

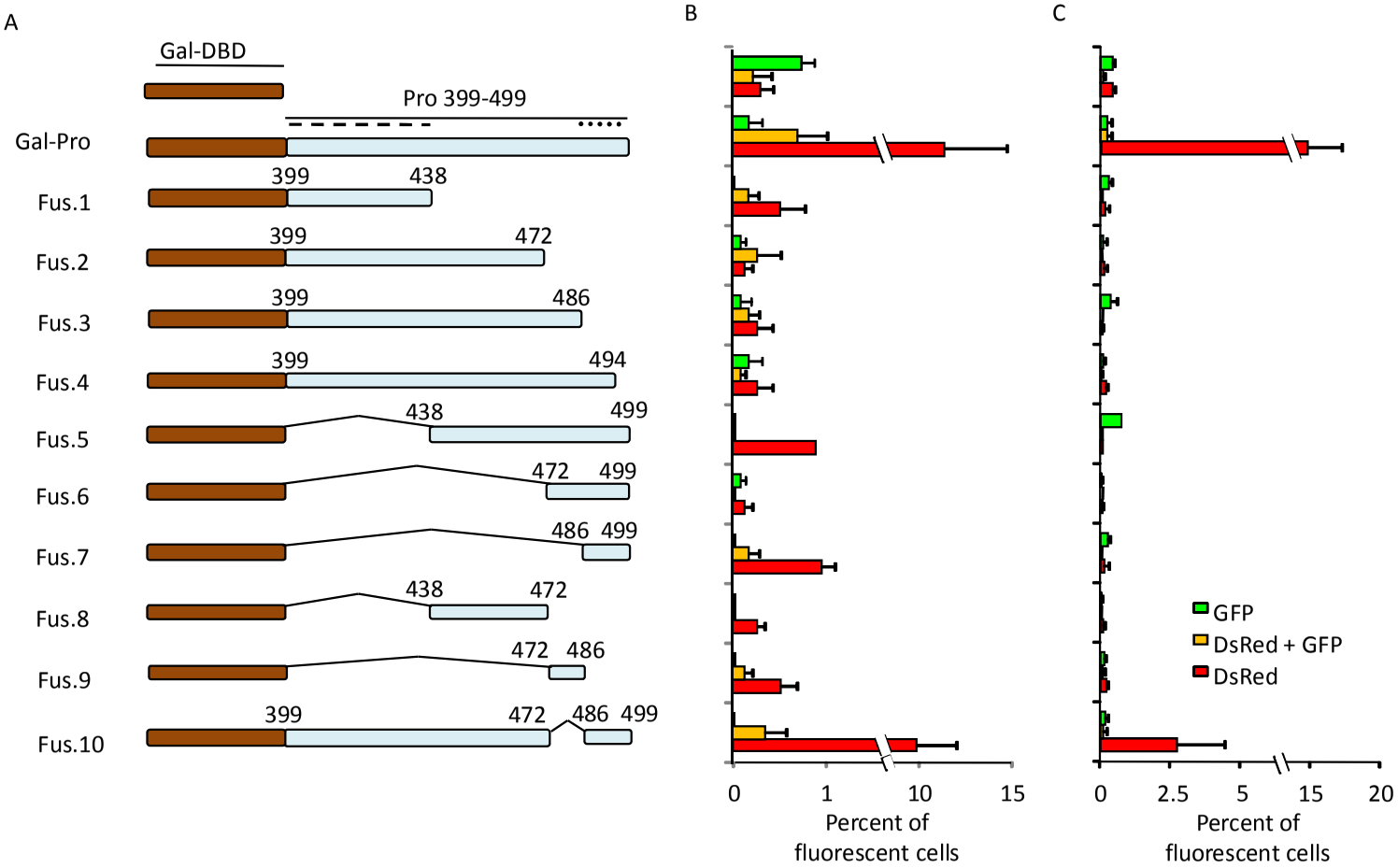
III.6. Supplemental material.



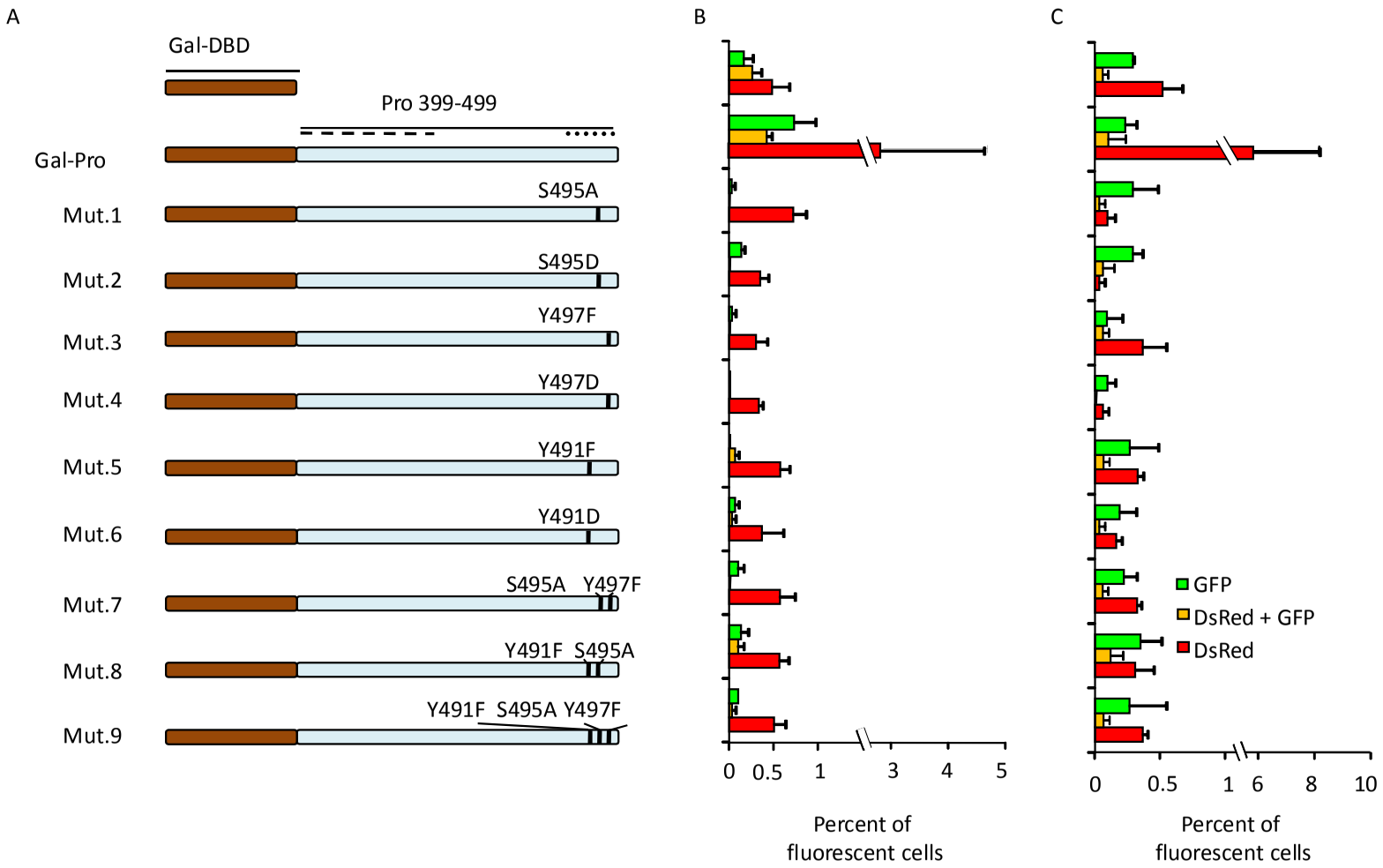
Supplemental Fig. 1. Localization of telomeric transgene integration in four stable cells clones.
(A) Metaphasic chromosomal spread of clone D17 were hybridized with fluorescent probes consisting of the GFP and DsRed sequences (red label) and the telomeric repeats (green label).
(B) Telomeric transgene integration of clones B09, B10, D17 and D34. Note the yellow color resulting from superposition of the green and the red colors, indicating colocalization of the insert and the telomere. The presence of two integrated chromosomes for the clone D34 results from the duplication of the chromosome after transgene integration. Transgenes of clones expressing higher levels of reporter proteins (B05, B23, D26 and D31) were also integrated at a telomeric position (data not shown).



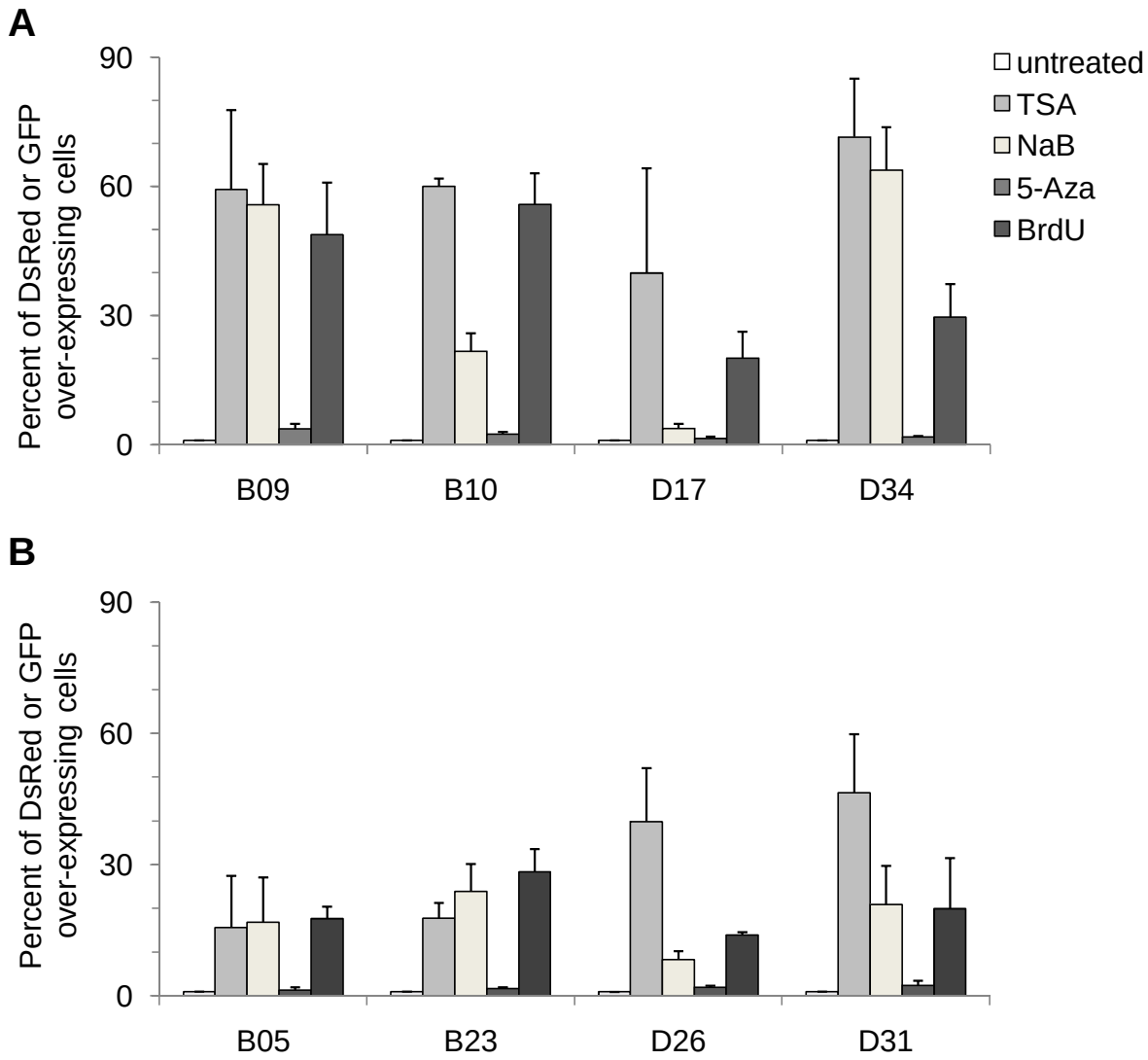
Supplemental Fig. 2. Non-targeted transgene integration in clones generated without telomeric repeats.
Integrations of transgene were analyzed as described for Fig. S2, using the clones generated with plasmid lacking the telomeric repeats as in Fig. S1C. Clones cB01 and cB17 (panels A and B) carry the convergent reporter construct, while clones cD06 and cD07 (panels C and D) carrying the divergently transcribed construct. Both cB01 and cB17 are integrated near or at the telomere, illustrating the frequent telomeric of subtelomeric integration of constructs, even when devoid or the telomeric DNA repeats, whereas cD06 and cD07 integrated the transgenes at internal chromosomal loci.



Supplemental Fig. 3. Deletions of the H3-interacting domains of CTF1 abolish its telomeric boundary activity.
(A) Schematic view of different deletion mutants are illustrated along location of the main H3 interaction domain (dotted line, amino-acids 486 to 499), and the accessory H3-interaction domain (dashed line, a.a 399-438), according to Muller and Mermod, 2000, as fusions to the GAL4 DNA-binding domain (Gal-DBD).
(B) Analysis of the GAL4-CTF1 fusions boundary activity in clone D17 as described in the legend to Fig. 2.
(C) Analysis of the GAL4-CTF1 fusions boundary activity in clone B09 as in panel B



Supplemental Fig. 4. Point mutations in CTF1 H3-interacting domains inhibit the boundary activity.
(A) Different mutations previously shown to decrease or abolish the histone H3 binding activity of Gal-Pro (Alevizopoulos et al., 1995) are illustrated as described in Fig. S4. The boundary activity of these constructs was analyzed in clone D17 (panel B) or B09 (panel C) as described in the legend to Fig. 2.



Supplemental Fig. 5. Telomeric position effects are relieved by histone deacetylation inhibitors.
The four clones with telomeric integration displaying low (A) or significant transgene expression levels (B) were treated with TSA (1µM), Na butyrate (NaB, 1mM), 5azadC (3µM) or BrdU (50µM). Unsilencing effects were assessed by recording the percentile of cells showing an increase of GFP and/or DsRed fluorescence as compared to the untreated cell populations shown in Fig S1. Both HDAC inhibitors (TSA and NaB) and BrdU treatment mediated transgenes unsilencing whereas DNA methylation inhibitor (5azadC) had no effect.

IV. CONCLUSIONS AND OUTLOOKS.

In the genome, regions separating permissive chromatin from silent chromatin are believed to be necessary to maintain a proper regulation of gene expression. Boundaries and insulators insure this function by separating chromatin domains of distinct structures and that may thus become crucial elements for the establishment of tightly controlled gene expression systems in biotechnology or in gene therapy.

The telomeric position effect has been described particularly in yeast, but how it occurs in higher eukaryotes remains partially unknown. In this work, the context of telomeric silencing was preferred to assess the boundary activity of Gal-fusion proteins, with the hope to guarantee the direction from which heterochromatin stems, and two mammalian cell lines were therefore assessed for their telomeric position effect. As shown by Baur *et al.* (2001), TPE occurs frequently and strongly in HeLa cells since low levels of reporter genes were correlated with telomeric insertions, even with the different reporter constructs. Although this phenomenon was less prominent in CHO cells, the low decrease in luciferase activity observed suggests that TPE may occur with a lower intensity and/or in a lower frequency in this cell line, but it does not exclude that telomeric position effect may be a widespread mechanism of silencing occurring at telomeres of most organisms from budding yeast to Human.

How telomeric position effect occurs in superior eukaryotes remains partially unknown and mechanisms modulating telomeric silencing are various and probably interacting between each others. Indeed, telomeric position effect occurs differently in function of the cell line analyzed and varies even among the cells from a same line, since some clones display only a partial silencing of telomeric transgenes while others are completely silenced. Such variegations may firstly stem from telomere length which is known to influence positively telomeric silencing, so that a clone over-expressing the telomerase or any gene involved in the ALT mechanism may exhibit a stronger telomeric silencing (Baur et al., 2001; Ning et al., 2003). Secondly, the system of telomeric integration described here allows an integration of transgenes at a telomere, but regardless of the chromosome identity. Consequently, the chromatin located at the centromere side of the reporter construct is able to influence positively or negatively its expression. Although chromosomal identification was assessed by FISH for all telomeric clones isolated in this work, chromosomal recombination and duplication occurring in HeLa cells made it difficult to compare the chromosome integrated with the human pattern of cytologic chromatin. Finally, histones marks present at

telomere appear as the most important element leading to telomeric silencing, and the complexity of the histone code may also affect importantly the variety of phenotype observed for the telomeric silencing. Histone deacetylation is largely involved in telomeric silencing, as previously observed in yeast. Indeed, according to Michishita *et al.*(2008), and consistent with the HDAC inhibitors assays described previously, the Sirtuin deacetylase family is thought to be the main responsible of histone deacetylation at telomeric chromatin, although other HDAC are also involved in this phenomenon. Nevertheless, ChIP experiments have shown that this deacetylation signal extends on a short range when compared to *S.cerevisiae*. Such difference might be explained by the fact that no self recruiting function has been yet discovered for the Sirtuin proteins in higher eukaryotes. Surprisingly, no histone mark involving lysine methylation among those tested here (including H4K20Me3, H3K27Me3 and H3K79Me2) nor HP1β were associated with gene silencing at telomeres (data not shown), suggesting that these histone marks and HP1β are not involved in the composition of mammalian telomeric heterochromatin. DNA methylation has been shown to occur in non-tumorous cell lines as an additive mechanism that could reinforce telomeric position effect (Pedram et al., 2006; Rincon-Arano et al., 2007), acting thus as an additive source of variegation of telomeric silencing. Nevertheless, methylation inhibition by 5azadC showed that DNA methylation was not involved in the telomeric silencing analyzed in this work.

As a first hypothesis, the polar effects of CTF1 on gene expression at telomeres might be explained by its transcriptional activity which would be facilitated at telomere distal locus. Indeed, in all clones carrying the reporter construct with diverging reporter genes, boundary activity of CTF1 remains more difficult to distinguish from its transcriptional activity, and a synergic effect on gene expression cannot be excluded. Nevertheless, clones carrying the converging reporter construct show that CTF1 acts even when distant from the promoter as expected from a chromatin remodeling or boundary activity. Moreover, asymmetrical activation of the gene distant from the telomere comforts this hypothesis when compared to VP16 activation that activates both reporter genes regardless of telomere orientation, but at a short distance only. Internal integrated controls give support to the boundary activity of CTF1 since it activates both reporter genes when they are not specifically inserted in a location adjacent to heterochromatin. Moreover, ChIP experiment has shown that histone marks associated with transcriptionaly active genes like trimethylated H3K36 were not increased by binding of CTF1 neither for GFP nor for DsRed, unlike what would be expected from a transcriptional activation mechanism (data not shown). Taken together, these data are fully

consistent with a chromatin boundary activity of CTF1 which is not dependent of its transcriptional activity.

How CTF1 protect genes from telomeric silencing has been attributed to the blocking of the spread of the SIR complex along the chromosome in yeast cells, but no such spreading has been yet described in mammalian cells, and the mammalian Sirtuin proteins have not been identified as chromatin components although they may be involved in the deacetylation of telomeric nucleosomes. Here, ChIP experiment showed that CTF1 blocks histone deacetylation signal spreading on a short range extent from the telomere, which is not exclusive with the possibility that this signal might be partly carried by SirT6 or SirT1 histone deacetylases. Nevertheless, binding of CTF1 between reporter genes is also associated with the increase of other histone marks at the telomere-protected *locus*, suggesting those are involved in the establishment of chromatin boundaries rather than in the composition of telomeric chromatin. Indeed, in the clone displaying an incomplete silencing of transgenes, trimethylated H3K9 and H4K20 were associated with restoration of histone acetylation, whereas they are generally observed in heterochromatic regions. On the other hand, in the clone displaying a complete silencing of transgenes, unsilencing of the telomere distal transgene was not associated with a recovery of histone acetylation but with the recruitment of the H2A.Z variant. This does not exclude that histone hypoacetylation is involved in telomeric position effect in this clone, since ChIP experiment confirmed low levels of acetylated histones and it was also responding to HDAC inhibitors. Nevertheless, lower levels of reporter genes expression in this clone suggests that H2A.Z recruitment may precede histone acetylation recovery during the establishment of chromatin boundaries, and this may explain why CTF1 mediating unsilencing can be associated with different chromatin modifications.

The establishment of chromatin boundaries involves many histones marks which may interact with one other. However, the possibility that CTF1 interacts with several chromatin modifying proteins remains unsubstantiated, thus CTF1 may recruit a histone mark that could lead to gene unsilencing by different ways. Two hybrids experiment have shown that CTF1 binds preferentially the H3.3 histone variant in mammalian cells (Ferrari et al., 2004), which is a variant known to be present at chromatin boundaries. Then, CTF1 boundary activity could be initiated by H3.3 recruitment, although up to now, no study has shown a link between H3.3 variant and the recruitment of H2A.Z or H3K9Me3. Although the H3.3 variant and H3 are different for a few amino acids, the analysis of Gal-Pro mutants shows that binding of CTF1

with histone H3 is needed for a correct boundary activity, so that its chromatin boundary activity may be directly dependent on the H3 binding function. CTF1 has been shown to act as a chromatin boundary at *S.cerevisiae* telomeres (Ferrari et al., 2004) and to exhibit an unsilencing activity in mammalian cells that may require its interaction with H3 histones (Pankiewicz et al., 2005). Thus, the present work links these two studies since it shows that the boundary effect of CTF1 is efficient mammalian cells, and that this boundary activity is dependant of the interaction of CTF1 with H3 histones. It remains difficult to determine which of the multiple histones marks may be involved or influenced by CTF1. However, recent studies in the laboratory using CHIP coupled to deep DNA sequencing have allowed to analyze genome wide histones modifications to determine what histone marks are preferentially associated to CTF1 binding sites (Milos Pjanic, personal comm.). Such study will permit to determine histone marks recruited by CTF1 at native CTF/NF1 binding sites in mouse fibroblasts and to give larger informations about the possible combinations of histone marks induced by CTF1-mediated boundary activity.

CTF1 displays interesting features that are required to isolate a transgene from its environment in biotechnological applications. Nevertheless, if CTF1 binding sites are able here to protect a transgene from silencing, it does not imply it protects it from activation as expected from a perfect insulator. Moreover, the use of native CTF1 binding sites in biotechnological applications leaves the problem of binding of other isoforms of the CTF/NF1 family strongly expressed in most of cells, and the possibility that they form heterodimers with CTF1, modulating negatively its activity. Consequently, insulating tightly a transgene from its chromatin environment would require probably the combination of several insulating elements. The plasmid construct described in this work permits to test Gal-fusion proteins on a large scale within the same clone, and consequently to compare these fusion proteins between each others. For instance, this system permitted to compare CTF1 boundary activity with other elements known as insulators and/or boundaries such as Gal-CTCF or Gal-USF1, which did not show any boundary activity in all clones analyzed (data not shown), even when both were bound between DsRed and GFP. Nevertheless, using Gal-fusion proteins in biotechnological applications remains limited since it needs to express also the Gal-fusion protein, but such two-reporters system may be ideal to screen for new boundary activities. Consequently, this system is currently being adapted to the study of other genomic putative boundary elements such as MARs and STARs. Such screening will permit to select genomic DNA elements displaying a strong boundary activity, before assessing their ability to control

gene expression *in vivo* when integrated in an inducible system. Moreover, this study will permit to determine the role of different MARs in regard to chromatin organization, as well as their possible recruitment of specific histone marks, which remains currently unknown. Finally, STARs were described as blocking the propagation of HP1-mediated silencing, so that testing these elements will permit to determine if other HP1 isoforms than HP1β acts in telomeric silencing in human cells.

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