Supplementary Results

Differentiation parameters of T2ECs

We followed the progression of differentiation by monitoring the surface expression of molecules characteristic of either immature or mature erythrocytes. As expected, a strong reduction in the expression of the immature-specific MEP26 antigen and an increase in the frequency of cells expressing the mature-specific JS4 antigen were detected after 48 hrs of differentiation (Figure S1, panel A). Furthermore, the percentage of haemoglobin expressing cells, as detected by benzidine staining, reached 30 to 40% after two days of differentiation (data not shown). Accumulation of haemoglobin more specifically reflected the transcriptional induction of βH and βA inside the β-globin cluster, as βH and βA transcripts increased 4 to 6 fold whereas embryonic gene transcripts increased only 2 fold (Figure S1, panel B). Cell cycle analysis by flow cytometry before and after 48 hrs of differentiation showed that both populations contained the same proportion of G1, S and G2 cells (Figure S1, panel C).

Supplementary Methods

Cell culture

Self-renewing T2ECs and differentiating T2ECs were generated from SPAFAS white leghorn chicken (PA12 line from INRA, Tours, France) as previously described (Damiola et al., 2004; Dazy et al., 2003; Gandrillon et al., 1999). Because large amount of cells were necessary to perform replication origin localization, we made slight modifications. Mainly exponentially self-renewing T2ECs were suspended at 3-6x10^5 cells/ml in the differentiation medium, which includes anaemic chicken serum (ACS). Different batches of Anaemic Chicken Serum (ACS) were prepared and tested for the minimal concentration required to induce robust cell differentiation, as monitored by blue acidic benzidine staining of haemoglobin as described (Quadrillion and Samara, 1998). To control for potential variability associated with ACS we decided to differentiate two independent preparations of T2ECs using two batches of ACS effective at very different concentrations (batch 1, 5% ACS; batch 2, 15% ACS). We observed that the replication initiation profile of both undifferentiated and differentiating cells was very reproducible (compare Fig 1A and 1C).

Flow cytometric analysis

We performed cell surface antigen detection using either MEP26 (early progenitors) or JS4 (late progenitors) antibodies as previously described (Dazy et al., 2003; Gandrillon et al., 1999). Cell cycle analysis was performed on cells washed in PBS, fixed in 75% cold ethanol, treated with RNAse at 1mg/ml and stained with propidium iodide at a final concentration of 50 μg/ml. Fluorescence data acquisition was done using a FACSCalibur flow cytometer and analysis was performed using the CellQuest program (both from Becton-Dickinson). MEP26 antibody was a kind gift of Dr Kelly McNagny.

Chromatin Immunoprecipitation

$25x10^6$ cells were fixed 10 min at room temperature with 0.1 volume of fixation buffer (PBS 11% formaldehyde 0.1M NaCl, 0.05M Hepes pH 7.9). Fixation was stopped by adding glycine at a final concentration of 0.125M. Cells were then washed twice in cold PBS. The pellet was suspended 10 min at 4°C in 2mL of lysis buffer (50mM Tris-HCl pH8.0, 10mM EDTA pH8.0, 1%SDS, 1/25 complete mini EDTA-free protease inhibitor cocktail (Boehringer)). The lysed cell suspension was frozen quickly in liquid nitrogen and kept at -80°C before chromatin sonication. Sonication conditions were established to shear DNA to an average size of 300 bp. Immunoprecipitation was performed overnight at 4°C in 1ml of 20mM Tris-HCl pH 8.0, 2mM EDTA, 150 mM NaCl and 0,25% Triton-X100 on an amount of chromatin corresponding to 10 μg of DNA using 5 μl of anti-diacetylated K9&K14 H3 antibodies (Upstate Biotech). Immunocomplexes were recovered and processed as described previously (Chen et al., 1999). Two independent immunoprecipitations were performed on two independent batches of undifferentiated or differentiating cells. Real-time quantitative PCR was performed by using a LightCycler and the QuantiTect SYBR GreenPCR Mix in accordance with the recommendations of the manufacturer (QIAGEN). Primer pair 5 was used as a standard in each PCR experiment.

Nascent strands preparation and quantification

Nascent strands were purified as described previously (Prioleau et al., 2003). Quantitative real-time PCR was performed by using a LightCycler and the QuantiTect SYBR GreenPCR Mix in accordance with the recommendations of the manufacturer (QIAGEN). Primer pair 24 was used as a standard in each PCR experiment. Primers were synthesized for each of the target sites to be amplified (table 1).

RNA isolation and cDNA synthesis

RNA extraction and reverse transcription assay were performed as previously described (Damiola et al., 2004; Dazy et al., 2003; Gandrillon et al., 1999). Sequences of the primers used for real-time PCR were the following: Beta-globin Adult GAACACCTTCTCCCAACTGTCC3’ and CTTTGGTGCTGGTGCTTAGTGG; Beta-globin Hatching TTTGCCCAGCTGAGCAAACTGC and CTGCCAGGCAGCCTGGCTGGCG; Beta-globin rho GAGAACTTCAGGCTCCTGGGGAAC and ACTTTCACACTGTGTCCTGCTCTGG; Beta-globin epsilon GAACACCTACGCCAAGCTGTCG and TTGGGGGCTATGGCCACGGCTG.

The ratio of expression (R) of each beta-globin gene was calculated as described (Damiola et al., 2004) using as a standard the invariant BBC1 gene (Dazy et al., 2003). The biological condition of reference was self-renewing T2ECs. The tested biological conditions were 24h or 48h differentiating T2ECs. Similar results were obtained with another invariant gene EF1 (data not shown) (Damiola et al., 2004).

Supplementary References

Chen, H., Lin, R.J., Xie, W., Wilpitz, D. and Evans, R.M. (1999) Regulation of hormone-induced histone hyperacetylation and gene activation via acetylation of an acetylase. *Cell*, 98, 675-686.

Damiola, F., Keime, C., Gonin-Giraud, S., Dazy, S. and Gandrillon, O. (2004) Global transcription analysis of immature avian erythrocytic progenitors: from self-renewal to differentiation. *Oncogene*, 23, 7628-7643.

Dazy, S., Damiola, F., Parisey, N., Beug, H. and Gandrillon, O. (2003) The MEK-1/ERKs signalling pathway is differentially involved in the self-renewal of early and late avian erythroid progenitor cells. *Oncogene*, 22, 9205-9216.

Gandrillon, O. and Samarut, J. (1998) Role of the different RAR isoforms in controlling the erythrocytic differentiation sequence. Interference with the v-erbA and p135gag-myb-ets nuclear oncogenes. *Oncogene*, 16, 563-574.

Supplementary Figure Legend

Figure S1: Differentiation parameters of T2ECs.

(A) Expression of differentiation antigens. T2ECs grown for 48 hours in self-renewing medium and for 24 and 48 hours in differentiating medium were analyzed by cell sorting for the expression of the immature specific MEP26 (black line) and the mature-specific JS4

antigens (Dark gray shaded profiles). Fluorescence profiles of cells incubated with FITC labelled goat anti-mouse secondary antibody only are indicated (Pale grey shaded profiles) as a control of staining. (B) Expression of embryonic (β-rho, β-epsilon, upper panels) and adult (βH, βA, bottom panels) globin genes in differentiating T2ECs. Quantifications of the β-globin transcripts were performed by real-time PCR on the same batches of cells (1 and 2 white numbers) as used in Fig. 1-3. The ratio of β-globin transcripts present in differentiating T2ECs (24h, pale grey; 48h dark grey) to transcripts present in self-renewing T2ECs are shown. Each PCR quantitation was performed in duplicate. (C) Cell cycle analysis. Histograms of propidium iodide (PI) staining intensity of self-renewing and differentiating T2ECs in batches 1 and 2 (white numbers).

Figure S2: 1-1.5 Kb Nascent enrichments in differentiated T2ECs confirm origins found with longer nascent strands:

1.0-1.5 Kb nascent strands prepared from differentiated cells (batch 1) were quantified by real-time PCR. The scale corresponds to fold of enrichments over the background. The abscissa scale is map position (nucleotide number). Black and gray arrows in the graph point towards constitutive and differentiation-activated origins, respectively. The positions of primer pairs 10, 20 and 30 are shown below and primer pairs 21', 23" and 29 are indicated inside the graph.

Figure S1

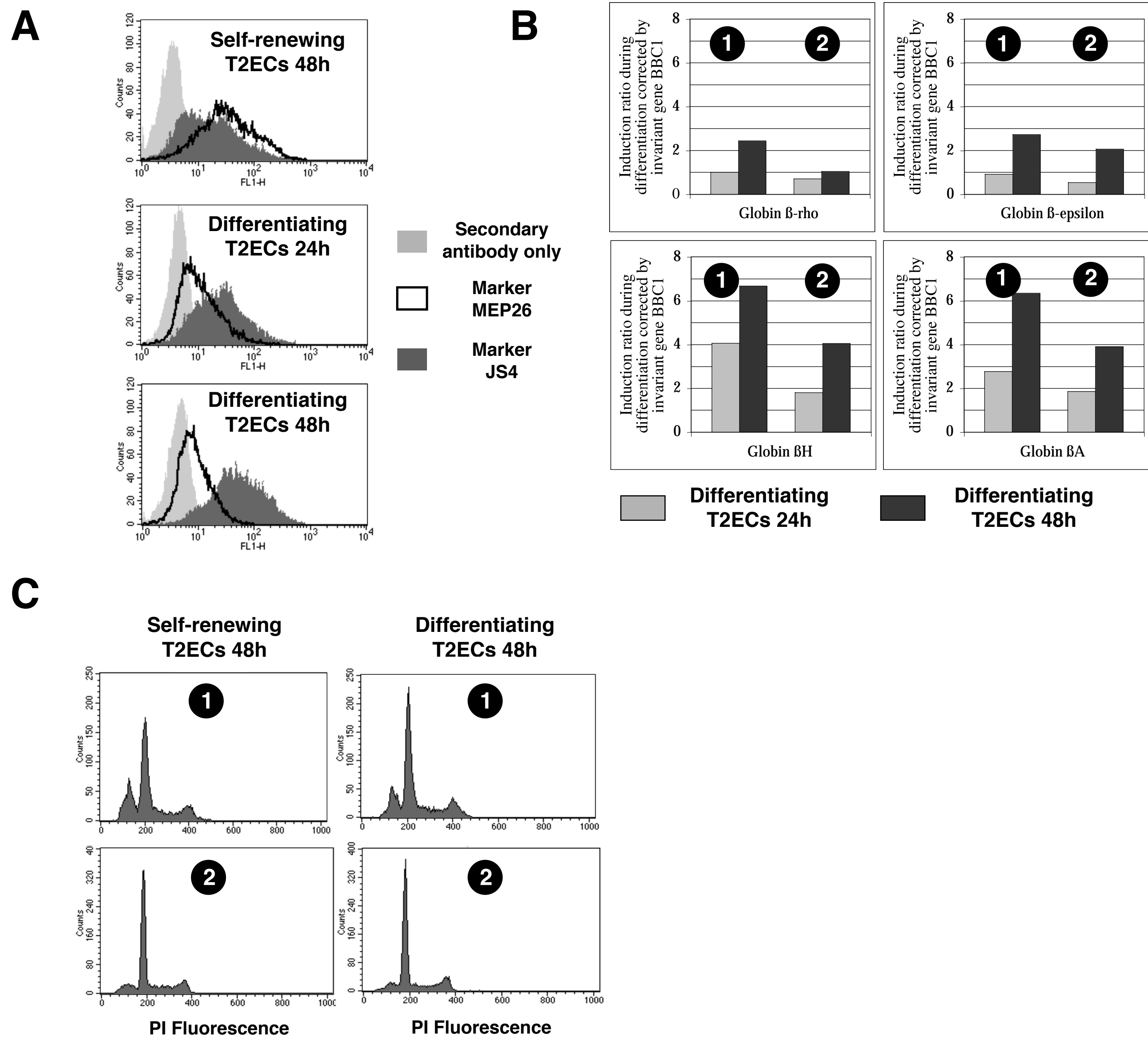
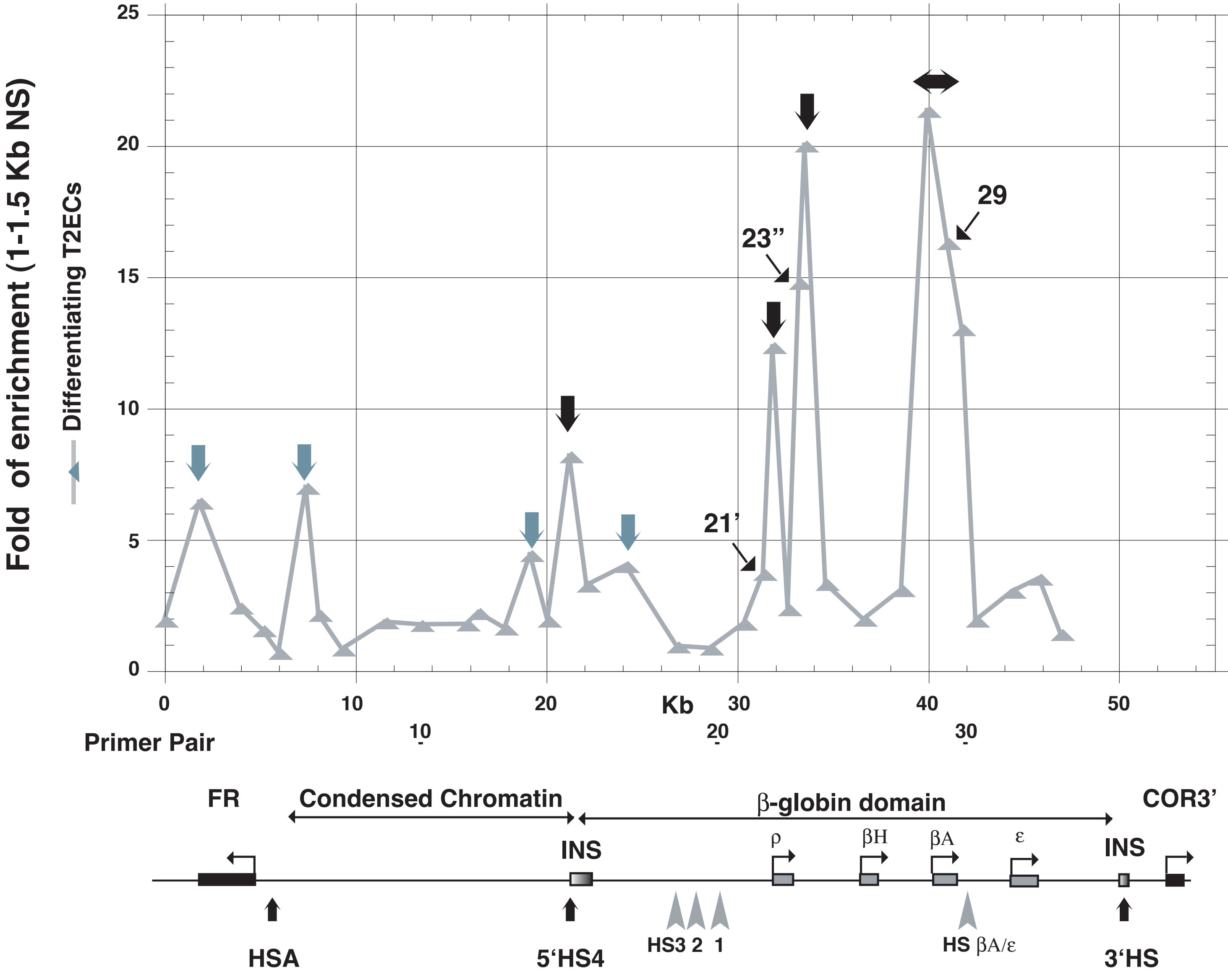


Figure S2



Supplementary Table 1 : List of primer sequences

Name Sequence 5' to 3'	Map position	Name Sequence 5' to 3'	Map position
Primers 1		**Primers 11**	
TGAGCTGCTGACTCTGCCCA	80	TGGCACCGATGGGATCACAT	15895
CATGTCCTCATGGCCAACAC	382	CACTGATACTGCAGCCTCTT	16189
Primers 2		**Primers 12**	
CAATGCAGCTCTGGCAGTCT	1904	GGTGTTCAATAATCAGACTGTC	16535
GTTACTCGTGTCCCTGCATG	2075	TCACATTTCAGCTGGCGCTC	16824
Primers 3		**Primers 13**	
CAGCAAGATGGTGTTGAAGG	4016	TCTCACTGTGCAGATGATGG	17905
CAGTGGTGTGGGTAATTGGA	4322	CATGGTGTCATCACAGCATC	18159
Primers 4		**Primers 14**	
GCCTCCTGACACAGCAGAGC	5217	GGATCCTGAAGCTGTTTGGC	19230
GCAGGTTTGAGGCAAGTTAG	5512	CTCTGCTGGGTTTCAGCAC	19363
Primers 5		**Primers 15**	
CTGAATGGTCTTTGTGGTCCC	6064	ACGGGATGGTGAAGGCACAG	20165
CTTGCTGTGGGAGACCTGCT	6348	TTAGCTCCCATTCCCCCACA	20472
Primers 6		**Primers 16**	
TGGGAGATAATTGTTAGCCAC	7499	GCCTCCAGGAAACCACTC	21423
TATGCTGGGAACAGCTATAC	7651	GACCTTCCAGGAAAGCCTG	21538
Primers 7		**Primers 17**	
GCCATACCCATTCACTCCAT	8166	GGACGTGGACATGCAGGTG	22200
AATGGGACACGCAGTCCCAG	8391	CCAGCCTTCATGATTTGACG	22508
Primers 8		**Primers 18**	
GCATCCTTCATCCAGCTG	9380	TGGGCAGTTTTCTAAGGGAA	24240
CAGTGAGCAGACTGTGAAGC	9533	GTGCTGGCAGGGCATTCCAA	24554
Primers 9		**Primers 18'**	
CTGTGGTCTGGTTTGTTTGG	11649	TGAGAGCTGAGCTGCTCCTG	24720
CAGCCTCTCCACTCATGCAC	11956	CAGACTGCAGCTGAAGGCA	24911
Primers 10		**Primers 19**	
CTGAGCTGTGACAGTGCC	13489	CAGCTCTGAGCACAGCACTG	26910
AATGGTCTTTGCCTGGGTCA	13652	CAGGGAATTCCTTCTGGGG	27216

Name	Map position
Sequence 5' to 3'	
Primers 20	
CTGTCTGAATATCCTGGCTC	28642
GTGATTCAATGTCAGGCACT	29 021
Primers 21	
GTGAGAGGGGCACTCCAGG	30441
GCAGTGCTCCGATAATGCC	30739
Primers 21'	
TGCACAGGGGCACCATTTTG	31444
CCTTGCATAAGGACAGCAGG	31610
Primers 22	
GAAGGGTGAGGGAAGTGCC	31941
TCAGTGTGCACAAGGTGTGG	32211
Primers 23	
GAACACCTACGCCAAGCTGT	32738
TGCTGCACCTTGTTCCACAC	32925
Primers 23'	
CAGCAGATGAAGGAGGGAAG	32920
GTCAGTTCCATGTCTGCCTG	33060
Primers 23''	
TTACCCCATTGCTCCCCTTC	33317
ACAGCCCTGAGCCCTCTTTC	33600
Primers 24	
GACGGTCAGGTTTGCCAAAG	33620
TCCTGAGGATACGTTTTTCAG	33887
Primers 25	
CTGGGAGCAAAGACACTGAC	34701
TGGTCACTCTGATTGCAGCA	34869
Primers 26	
GATACGCACTGAGCTCTCGT	36700
CACCCATGATCTCGTAGCCA	37000
Primers 27	
GAACAAGTCATTGCACAACGG	38690
GGCAGTGAAACCAAGTGCTC	38994

Name	Map position
Sequence 5' to 3'	
Primers 27'	
GACAGAGCACTTGGTTTCAC	38970
GATGCTGCGAGCCACATCTG	39208
Primers 27''	
GAGTGCTGTGGTTTGGAACT	39870
CAGCTCTGCAGCTCTATAC	40010
Primers 28	
TGGTGTGGCCACGGATCTG	40040
GTGATGAGCTGCTTCTCCTC	40333
Primers 28'	
GAGGAGAAGCAGCTCATCAC	40310
GTGTACACCTTCAACTGCAC	40453
Primers 29	
CATAGAGCAAGGGACGGTG	41070
TACTGTGGGAAGAGTAGCTC	41231
Primers 30	
AGCGCTTTGTGCTCAGTGG	41835
ATGACTCCTCTGTGTCAGTGA	41989
Primers 30'	
TCCCCTGACTCACTGCTGG	41835
ACACGGAGTGTCCTCCTATG	42700
Primers 31	
CATAGGAGGACACTCCGTGT	42681
TACCCTTTTCCTTCCGGCTG	42857
Primers 32	
GCAGCTCCGCTCCAAGCTCT	44490
GGCTGGAGAGGTTCCCAAAG	44810
Primers 33	
TCCAAGCAGCACTAACCCTG	45900
ACGTTGACCAGCTTCTGCCA	46039
Primers 34	
GCTGAATGCTGTGCCTCTGG	47020
CCATCAACCTGCTAGAGAAG	47318

Primers 35
CCCAGATTTGCTTCATCAGGAG 48560
GAGATGTGATAACAGACTGCCA 48758

Primers 36
CACTTCTGCTCTACAAGGCC 49950
ACTGACATGGAAACACACGG 50103

Primers 37
GCAGTGCAGACCCATCCCT 51170
TCTTTCTGTGCGTCATCAGC 51455

Primers 38
CCAAGCAAGTCCAGACAGAG 52817
GCAAGCCTACATTCCTCCC 52996

Primers 39
TCATGAACTCCCAGTACCAG 54424
TGCACACTGATCAGTAGGTC 54567

Primers LYS :
CGGGTATCATTAGTGCCGAG
CTGCCAGTATATCCTGGCAAA