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(71) Applicant *(for all designated States except US)*: **THE UNIVERSITY COURT OF THE UNIVERSITY OF GLASGOW** [GB/GB]; Gilbert Scott Building, University Avenue, Glasgow, Lanarkshire G12 8QQ (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **WILSON, Joanna, Beatrice** [GB/GB]; I.B.L.S Division of Molecular Genetics, Robertson Laboratory, University of Glasgow, 54 Dumbarton Road, Glasgow, Lanarkshire G11 6NU (GB). **REPELLIN, Claire, Evangeline** [FR/GB]; I.B.L.S Division of Molecular Genetics, Robertson Laboratory, University of Glasgow, 54 Dumbarton Road, Glasgow, Lanarkshire G11 6NU (GB).

(74) Agents: **FORREST, Graham** et al.; Mewburn Ellis LLP, York House, 23 Kingsway, London, Greater London WC2B 6HP (GB).

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(54) Title: TISSUE-SPECIFIC TRANSCRIPTION BY RNA POLYMERASE III

(57) Abstract: The invention provides materials and methods which enable the tissue-specific transcription of desired DNA sequences by RNA polymerase III. Transcriptional regulatory elements which direct tissue specific transcription by RNA polymerase II are combined with transcriptional regulatory elements from promoters which direct transcription by RNA polymerase III, to achieve tissue specific transcription by RNA polymerase III. Constructs comprising such hybrid promoters are particularly useful for expression of RNA molecules such as siRNAs and ribozymes.

Tissue-specific transcription by RNA polymerase III

Field of the Invention

The invention relates to eukaryotic gene transcription, and in particular to the tissue-specific transcription of genes by RNA polymerase III.

Background to the Invention

Mammalian cells contain three RNA polymerases. These are designated RNA polymerase I (pol I), RNA polymerase II (pol II) and RNA polymerase III (pol III). Pol I synthesises rRNAs, which are the precursors of the main ribosomal RNAs. Pol II transcribes all protein-encoding genes leading to a messenger RNA (mRNA). Pol III synthesizes 5s rRNA, tRNAs, 7 SL RNA, U6 SnRNA and a few other small stable RNAs.

The regulatory sequences of pol II genes often include an enhancer, which is usually located upstream of the coding sequence or alternatively in introns or even 3′ of the coding sequence itself. An enhancer is composed of DNA sequence motifs which bind transcription factors to facilitate the transcription of pol II genes. These transcription factors may be present in all cells or only in some specific cell types. Thus enhancers can give tissue specificity to pol II transcription. For example the immunoglobulin heavy chain (IgH) enhancer Eμ directs the expression of heterologous genes to the B cell compartment.

Another classical feature of a pol II gene is the presence of a TATAA box, which is located approximately 30bp upstream of the start site of the transcript and enables initiation at the correct site. Pol II genes may or may not have introns, and all have a 3′ AATAAA polyadenylation (pA) signal which triggers the addition of 80 to 250 adenylate residues at the end of the mRNA. Termination signals can also be found at the end of some pol II genes.

The most striking and unusual feature of the promoters used by RNA pol III is that the majority require sequence elements downstream of the RNA start site within the transcribed region. These internal control regions are generally discontinuous structures composed of essential DNA sequence blocks separated by non-essential regions (8). Three basic types of promoter are used by RNA pol III, designated types I, II and III.

The type I promoter requires three internal elements for efficient transcription: an A block, an intermediate element (IE) and a C block. This promoter arrangement is unique to 5s RNA.

The most common promoter arrangement used by RNA pol III is found in the tRNA genes and is the type II promoter. It consists of two highly conserved sequence blocks, A and B, within the transcribed region. The A block of type I and II promoters are homologous. The location of block B is variable.

A minority of vertebrate RNA pol III templates lack any requirement for intragenic promoter elements. These are referred to as type III promoters and require a TATA-like box, a proximal sequence element (PSE) and a distal sequence element (DSE) for efficient expression. All the pol III genes are terminated by a TTTT termination signal (with no pA tail). The expression of a pol III transcript is usually constitutive and not tissue specific. The pol III RNAs are short and non-coding. Therefore this makes pol III promoters ideal for vectors where expression of short non-coding RNA is required. For example, the H1 pol III promoter is used in vectors for the generation of double stranded RNA (dsRNA) hairpin-loop structures, such as the pSilencer vector from Ambion.

Meissner et al. (7) have described an attempt to modulate pol III expression from a type III promoter. They mutated the TATA-like box of a human U6 gene promoter so that it was incapable of binding the endogenous transcription factor TBP but acquired the ability to bind a mutant form of TBP. However, this approach is

limited by the need to supply the mutant TBP into the expression system.

With RNA interference (RNAi) use and therapy burgeoning, it is highly desirable to be able to express pol III type transcripts, but in a controlled and tissue specific fashion. To date this has not been achieved.

EBER1 and EBER2 are non-polyadenylated, non-translated RNAs of Epstein-Barr virus (EBV). The EBER genes are transcribed by pol III (9) but do not fall neatly into any of the 3 pol III promoter types. For instance, they have A and B blocks which are characteristic of type II promoters and are essential for transcription (See figure 1). However, efficient transcription of the EBER genes also requires 3 upstream elements: a TATA-like box (TGTA, -28 to -23, relative to the start site for transcription for EBER1) an ATF binding site (-55 to -40 for EBER1) and an Sp1 binding site (-77 to -56 for EBER1). The ATF and Sp1 sites are usually associated with sequences transcribed by RNA polymerase II (5) Successive deletions of the 3 upstream elements showed a significant reduction in EBER1 transcription (10), thus suggesting that both pol II and pol III elements are necessary for optimal EBER transcription.

Summary of the Invention

The present inventors have surprisingly found that tissue-specific RNA pol III transcription may be achieved by combining RNA pol III regulatory elements with transcriptional regulatory elements normally associated with pol II genes.

Thus the present invention provides a nucleic acid molecule comprising an expression cassette, the expression cassette comprising a target sequence to be transcribed by RNA pol III operably linked to RNA pol III transcriptional regulatory elements, said expression cassette further comprising a pol II transcriptional regulatory element which provides selective transcription of genes by RNA pol II in a target cell or tissue,

whereby said target sequence is selectively transcribed by RNA pol III in said target cell or tissue.

The nucleic acid molecule (or "construct") of the invention is typically a DNA molecule. It comprises a target sequence to be transcribed into RNA by RNA pol III. The target sequence will typically be operably linked at least to one or more pol III promoter elements and a pol III terminator element in order that the target sequence can be transcribed by pol III.

In addition to the pol III promoter element(s), the nucleic acid molecule further comprises a pol II transcriptional regulatory element. The pol II element is capable of conferring tissue-specific expression on genes transcribed by RNA pol II. The present inventors have found that, by combining such a regulatory element with a pol III promoter, tissue specific pol III transcription may be achieved.

Preferably, the pol II transcriptional regulatory element is a pol II enhancer element. For example, it may bind to a transcription factor capable of stimulating transcription of the target sequence by pol III in the target cell or tissue. In preferred embodiments, the transcription factor is endogenous to the target cell or tissue.

The enhancer may provide B cell specific expression. For example, the enhancer may be an immunoglobulin Eμ enhancer. The Eμ enhancer used in the constructs described in the Examples was first described in reference 17 and has the sequence:

AGCAGCTGGC AGGAAGCAGG TCATGTGGCA AGGCTATTTG GGGAAGGGAA AATAAAACCA
CTAGGTAAAC TTGTAGCTGT GGTTTGAAGA AGTGGTTTTG AAACACTCTG TCCAGCCCCA
CCAAACCGAA AGTCCAGGCT GAGCAAAACA CCACCTGGGT AATTTGCATT TCTAAAATAA
GTTGAGGATT CAGCCGAAAC TGGAGAGGTC CTCTTTTAAC TTATTGAGTT CAACCTTTTA
ATTTTAGCTT GAGTAGTTCT AGTTTCCCCA AACTTAAGTT TATCGACTTC TAAAATGTAT
TTAG

Variants of this sequence may be used, e.g. having at least 60%, 70%, 80%, 90% or 95% sequence identity to this sequence, as long as the variant enhancer sequences retain the ability to direct transcription of an operably linked sequence preferentially in B cells.

The RNA pol III transcriptional regulatory elements of the construct may consists entirely of intragenic or downstream elements, located downstream of the transcriptional initiation site. For example, the construct may comprise an A block. Typically, the construct further comprises either a B block or C block. Constructs comprising an A and C box preferably also comprise an Intermediate Element (IE) located between the two.

Optionally, the pol III transcriptional regulatory elements may further comprise one or more upstream elements, which may include a TATA-like box.

Alternatively, the pol III regulatory elements may consist entirely of upstream elements. For example, the construct may comprise at least a TATA-like box. Typically, the construct will also comprise either a Proximal Sequence Element (PSE), a Distal Sequence Element (DSE), or both.

The pol III regulatory elements may comprise some or all elements of an EBER promoter. Preferably these include the A and B blocks (located downstream of the initiation site), and preferably also the TATA-like box (located upstream of the initiation site). The Sp1 and/or the ATF binding sites may also be included as required.

Examples of suitable EBER promoter elements include:

A block: CGCTGCCCTAGAGGTT
B block: GGGTACAAGTCCC
TATA-like box: TGTAGA
Sp1 binding site: CCGCCC

ATF binding site: TGACG

These sequences are used in the constructs described in the Examples below. It will be understood that a certain amount of variation in these sequences may be tolerated without abrogating transcription by RNA polymerase III.

The target sequence may encode a RNA molecule capable of inhibiting the expression of a target gene in the same cell. For example, the encoded RNA molecule may be an antisense RNA molecule, RNAi molecule or ribozyme.

In a specific embodiment, the encoded RNA molecule is capable of inhibiting EBER expression in an Epstein Barr Virus (EBV)-infected cell, and is expressed specifically in B cells.

Such constructs may be used for the treatment of conditions associated with EBV infection, and in particular with EBV-associated tumours. These include Burkitt's lymphoma (BL),which is common in children in equatorial Africa, and nasopharyngeal carcinoma (NPC), which is common in adult men in Southern China and in ethnically related populations. (In respect to the size of the Chinese population, NPC is regarded by WHO as a major world health problem).

Other EBV-associated cancers of the Western world include Hodgkin's disease (HD) and certain AIDS related B-cell lymphomas. In these EBV-infected tumours, the virus expresses different genes. In all tumour cells described, two small non-coding, RNA pol III transcribed genes called EBER1 and EBER2 are expressed. It is believed that the function of these genes in viral infection is a defence mechanism against the immune system, inhibiting interferon induction, thereby allowing infected cell survival. In this regard, it has been shown that EBER1 binds to and inhibits PKR, a protein kinase capable of inducing interferon synthesis when activated by double stranded RNA.

Thus the invention provides the use of a nucleic acid as described above in the preparation of a medicament for the treatment of a condition associated with EBV infection, and in particular for the treatment of EBV-associated cancers.

Also provided is a nucleic acid construct as described herein for use in a method of medical treatment. This aspect of the invention is not limited to the treatment of conditions associated with EBV infection, but extends to any condition which may be treated by expression of a suitable RNA (e.g. an antisense, RNAi, siRNA or ribozyme molecule) in a target cell.

In a further aspect, there is provided an expression vector comprising a nucleic acid construct as described herein. The vector may be a linear molecule (e.g. one designed for homologous recombination into a host cell chromosome), a plasmid or a viral vector (e.g. a retroviral or adenoviral vector).

In a further aspect the invention provides a cell comprising a nucleic acid or an expression vector as described herein.

Also provided is a non-human transgenic animal comprising a nucleic acid or an expression vector as described herein.

The invention further provides a method of modulating transcription of a target sequence operably linked to RNA pol III transcriptional regulatory elements, the method comprising operably linking said target sequence to a pol II transcriptional regulatory element which provides selective transcription of genes by RNA pol II in a target cell or tissue, whereby said target sequence is selectively transcribed by RNA pol III in said target cell or tissue.

For example, the method may be performed by introducing a pol II regulatory element into the chromosome of a cell, e.g. by homologous recombination. The target sequence may, for example, be a gene endogenous to the cell which is already transcribed by

pol III. Introduction of the pol II regulatory element then modulates expression of that gene, such that it is transcribed in a tissue-specific manner.

The method may further comprise the step of generating a non-human transgenic animal from the resulting cell.

Description of the Drawings

Figure 1 shows three variant constructs comprising an Eμ enhancer linked to the EBER1 gene. Constructs A and B comprise all regulatory sequence elements found in wild type EBER1, including the Sp1 binding site, ATF binding site and TGTA box located upstream of the transcriptional initiation site, and the A and B boxes located downstream. In construct A (p670), the Eμ sequence is located approximately 245 nucleotides upstream of the Sp1 site, while in construct B (p671) the two elements are adjacent. In construct C (p672), the Sp1 and ATF sites are deleted and the Eμ enhancer is placed adjacent the TGTA box. All three constructs have been shown to express in B-cells in culture. Tissue-specific expression of construct B has been confirmed *in vivo* in transgenic mice. Transgenic mice carrying constructs A and C have also been successfully generated and confirmed to express the respective constructs.

Figure 2 shows a Northern blot performed with 12μg of total RNA derived from transiently transfected 39.415 cells 72 hours after transfection. The blot was probed with EBER1 (top blot) and rpL32 (a mouse-specific probe) to control for loading (bottom blot). Expression can be seen from both the linear constructs and the supercoiled constructs, except for supercoiled p672 where no RNA was loaded on the gel (as shown by the rpL32 loading control). It was subsequently shown that linear 672 does express in culture.

Lane 1= no DNA transfected
Lane 2=p669 control
Lane 3=p670 linear

Lane 4=p671 linear
Lane 5=p672 linear
Lane 6=p661 supercoil
Lane 7=p669 supercoil
Lane 8=p670 supercoil
Lane 9=p671 supercoil
Lane 10=p672 supercoil
Lane 11=Raji (EBV+)
Lane 12=IB4 (EBV+)
Lane 13=BJAB (EBV-)

Figure 3 shows a Northern blot of mRNA extracted from mice positive and negative for the p671 transgene. Tissues from 4 month old positive and negative siblings were collected. RNA was extracted from the different positive and negative lymphoid tissues as described in section 4 of experimental procedures. For small tissues, such as MLNs, PLNs, and Peyer's patches, tissues from 2 positive and 2 negative animals were pooled for the RNA extraction. DNAse I treatment was performed on 10µg of RNA followed by acid phenol extraction. An RT-PCR was performed on 2µg of RNA as described in section 4 of experimental procedures. A control for the PCR reaction was performed using an EBER1 plasmid as template. Half of the RT-PCR products was electrophoresed through an agarose gel followed by Southern blotting. For the PCR control, one tenth of the reaction was used. The Southern blot was hybridised with an EBER1 probe. In the negative tissues, faint background signal may be seen. In the transgenic positive tissues, expression is evident in spleen, BM, MLNs, PLNs, P.Patches and thymus, but not in liver or other tissues (data not shown).

Figure 4 illustrates the use of p670 (see Figure 1) to carry a siRNA capable of targeting EBER 1. The transcribed EBER 1 sequence of p670 is replaced with a sense sequence targeting the start and A box of EBER 1, followed by a loop and an antisense sequence. A schematic diagram is shown in Panel A. The sequence itself is shown in Panel B. Transcribed sequence derived from

EBER1 is underlined. Within the transcribed region, the B box of EBER1 is shown in italics. The siRNA sequence is shown in normal capitals; the bold font shows those parts of the siRNA sequence which will hybridise to one another Upstream of the transcribed region, the Sp1, ATF and TGTA box of EBER1 are shown in italicised capitals. The sequence of the Eμ enhancer is shown in Panel C.

Detailed Description of the Invention

Pol III promoters

RNA pol III is located in the nucleoplasm and synthesises the precursors of 5S ribosomal RNA, tRNAs and other small nuclear and cytosolic RNAs. For a review of the RNA pol III transcriptional apparatus, see ref. 14.

The pol III promoter elements used in the constructs of the invention may be derived from the promoter of any gene transcribed by pol III. These include, but are not limited to, type I, II and III pol III promoters. A naturally-occurring pol III promoter or a portion or fragment thereof may be used. Alternatively, a pol III promoter may be assembled de novo using individual promoter elements.

A type I promoter typically comprises an A block, a C block and an Intermediate Element (IE) located between them. All of these elements are located intragenically, i.e. downstream of the transcriptional initiation site. Type I promoters are found, for example, in 5S ribosomal RNA genes from Xenopus laevis and Drosophila. They are thought to be unique to 5S rRNA genes.

A typical type II promoter comprises an A block and a B block, also located intragenically, i.e. downstream of the transcriptional initiation site. The A block is homologous to that of the type I promoter; the location of the B block is variable. Type II promoters are the most common pol III promoter

type. Examples can be found in most tRNA genes, adenoviral VA genes, and many families of eukaryotic repetitive sequence including Alu repeats.

A typical type III promoter comprises a TATA-like box, a Proximal Sequence Element (PSE) and a Distal Sequence Element (DSE), all of which are located upstream of the transcriptional start site. Examples include U6 snRNA genes. The most well-characterised type III promoter is associated with a human U6 snRNA gene.

Other types of pol III promoter exist which are not readily placed in any of the categories described above. Example include the EBV EBER 1 and 2 promoters. These promoters possess intragenic A and B blocks, as seen in type II promoters, which are essential for transcription. They also possess three elements upstream of the transcriptional start site, namely a TATA-like box (TGTA), an ATF binding site and an Sp1 binding site.

Thus a pol III promoter in a construct of the present invention may have numerous configurations. For example, it may consist entirely of elements located downstream of the transcriptional start site. These elements may be intragenic, or may extend downstream of the transcriptional termination site, particularly when the transcript is relatively short. Alternatively, they may consist entirely of upstream elements, or may comprise a combination of both element types.

For example, a promoter consisting entirely of downstream regulatory elements normally comprises at least an A block. Typically, it further comprises either a B block or C block. Constructs comprising an A and C box preferably also comprise an Intermediate Element located between the two.

A promoter comprising both downstream and upstream elements typically comprises some or all of the elements of an intragenic promoter as described above, in combination with one or more

upstream elements, which may include a TATA-like box, a PSE and/or a DSE.

Alternatively, a promoter may consist entirely of upstream elements. Such promoters preferably comprise at least a TATA-like box. They may also comprise either or both of a PSE and a DSE. Additionally or alternatively, A, B and/or C blocks may be functional when placed upstream of the transcriptional start site.

The skilled person will appreciate that, in order to obtain the desired level of transcription by pol III, it may be necessary to take other factors into account. These include the relative spacing of the various promoter elements, as well as their absolute distances from the transcriptional start site. Details, and examples of particular promoters which may be used in the methods and constructs described here, may be found (for example) in refs. 8 and 14, and references cited therein.

It will be apparent that the precise configuration of the promoter is not critical for the present invention, as long as the target sequence is capable of being selectively transcribed by pol III in the target tissue or cell type when the promoter is supplemented by the pol II regulatory element. By "selectively transcribed" is meant that the transcription is specific to that tissue, rather than being constitutive, throughout all (or substantially all) cells or tissues of the organism.

The construct will also contain other features necessary for pol III transcription, such as a pol III termination site. Typically, a string of 4 or 5 U residues in the transcript is sufficient to serve as a termination signal.

Pol III transcripts are not polyadenylated and typically do not encode proteins. Thus they are readily distinguished from pol II transcripts, which are all polyadenylated apart from histone-encoding transcripts.

Whether a transcript is produced by pol III or pol I can be determined by testing the sensitivity of transcription to the presence of α-amanitin. Pol III transcription is inhibited by α-amanitin (as is pol II transcription); however pol I transcription is unaffected by α-amanitin.

Pol II elements

Genes transcribed by pol III are generally expressed constitutively, in substantially all cells and tissues. The present invention provides tissue-specific pol III transcription by adding a pol II transcriptional regulatory element to the basal pol III promoter. Thus, in addition to the pol III promoter, the constructs of the invention comprise a pol II regulatory element.

Like pol III, pol II is also located in the nucleoplasm. However, pol II is responsible for synthesising the precursors of all mRNAs from protein-coding genes, as well as some small nuclear RNAs. Pre-mRNAs generated by pol II are processed by cap formation, polyadenylation and splicing, as is well known to those skilled in the art.

Genes transcribed by pol II have a number of regulatory sequences which control initiation of transcription. Many pol II promoters have a TATA box (consensus sequence TATA(A/T)A(A/T)) located 25 to 30 bases upstream of the initiation site. Some genes instead possess an initator element overlapping the transcriptional start site. These elements are required for formation of the transcription complex and initiation of transcription. Other sequence elements are often required for efficient pol II transcription. These are often located within 100 to 200 bases of the initiation site. Examples include the SP1 and CCAAT boxes, as well as the ATF box found in the EBER promoters. Together with the TATA box or initiation element, these may be regarded as the pol II promoter. These generally do not control

transcription in a tissue specific way and are generally not suitable for use as pol II regulatory elements in the context of the present invention.

Many pol II genes also possess other regulatory elements, including repressor and enhancer sequences. Certain of these elements confer tissue-specific regulation on the transcription of the genes to which they are linked. Most if not all tissues express structural genes characteristic of themselves, which are either not expressed, or are expressed only at very low levels, in other tissues. Their transcription by pol II is controlled by tissue specific regulatory elements. For example, the human immunoglobulin heavy chain enhancer Eμ is capable of directing pol II transcription of a gene specifically in the B cell compartment.

Thus the pol II elements used in the constructs of the invention are capable of conferring tissue-specific expression on a gene transcribed by pol II. Numerous examples of such elements are known.

By "tissue-specific transcription" or "tissue-specific expression" is meant that the transcription occurs at significant levels in one or more particular ("target") cell or tissue types, but at low levels or not at all in others. Preferably the level of transcription in the target cell or tissue type is at least 5 fold, and preferably at least 10, 100 or 1000 fold higher than the level of transcription in one or more other cell or tissue types of the organism. More preferably, the level of transcription is at least 5, 10, 100 or 1000 fold higher than the level of transcription in substantially any other cell or tissue type of the organism.

Preferably, transcription of the target sequence is not detectable (above background levels) in cells or tissues other than the target type.

The level of transcription may be determined by Northern blot, RT-PCR, in situ hybridisation or any other appropriate technique.

The pol II element may function in a number of different ways. It may repress or inhibit transcription in cells or tissues other than the target cell or tissue type. In such cases the pol III promoter may be capable of driving constitutive pol III transcription of the target sequence in the absence of the pol II element. Such elements may be regarded as repressors.

Alternatively, the pol II element may stimulate transcription in the target cell or tissue type. For example, it may bind to one or more transcription factors expressed preferentially (or exclusively) in the target cell or tissue. Thus the pol II element increases the basal level of expression from the pol III promoter.

In preferred embodiments the pol III promoter is constructed such that the pol II element is required for pol III transcription to occur. Thus a pol III promoter is used which is insufficient to drive pol III transcription in the target cell or tissue in the absence of the pol II element, but where the combination of pol III promoter and pol II element is capable of driving pol III transcription.

Alternatively, the pol II element may work by a combination of the above mechanisms. Thus, it may inhibit transcription in cells and tissues other than the target cell or tissue, while also stimulating transcription in the target cell or tissue. Without wishing to be bound by any particular theory, it is believed that the Eμ enhancer works in this way, inhibiting expression in non-lymphoid tissue while stimulating expression in lymphoid tissue.

AP1 binding sites are found in many pol II enhancers. Binding of a c-Jun/c-Fos dimer (expressed in certain cells) to the site may result in transcriptional activation of a linked gene. By

contrast, binding of JunB (expressed in different cells) in place of c-Jun may lead to repression.

Pol II elements which stimulate transcription in the desired tissue/cell type (whether or not they also inhibit transcription in other cells and tissues) may be regarded as pol II enhancers.

The skilled person will be aware that tissue specific pol II regulatory elements, such as enhancers, contain binding sites for multiple factors which interact in complex ways in order to achieve tissue-specific transcription. Thus it will be understood that the pol II regulatory elements for use in the present invention may be composed of a plurality of binding sites capable of binding to a number of different protein factors. The term "pol II regulatory element" should be construed as referring to the minimal nucleic acid sequence required to confer tissue specific expression on a gene transcribed by pol II.

The pol II element may be endogenous to the cell. That is to say, at least one copy of the element is found elsewhere in the genomic DNA of the cell (or in the wild type genomic DNA of the species from which the cell is derived), operably linked to a gene transcribed by pol II to provide tissue-specific expression of that gene. However it will be appreciated that pol II elements which are not endogenous to the cell may also be used. For example, enhancer elements from other species may be used, as these elements often display a high level of evolutionary conservation and so function in other species. Mutants or derivatives of naturally-occurring sequences may also be used as long as they retain their function. Pol II elements derived from viruses (e.g. the cytomegalovirus enhancer) may also be useful in the constructs of the invention; transcription of viral genes in host cells often shows remarkable tissue specificity. Prokaryotic elements may also be linked to pol II elements in order to confer conditional expression.

Where the pol II element binds one or more transcription factors which stimulate transcription of the target sequence, that transcription factor is preferably endogenous to, and naturally expressed in, that cell or tissue (i.e. the transcription factor is expressed there without the need for genetic manipulation).

Uses of constructs of the invention

The constructs of the invention may be used in any context in which it is desirable to produce a pol III transcript in a tissue-specific manner.

Pol III termination is highly specific, and does not result in a polyadenylated transcript, unlike the majority of pol II transcripts. These properties make pol III transcripts extremely useful, for example, as antagonists of gene expression. Such antagonists typically have one or more stretches of sequence complementary to the DNA encoding the gene to be downregulated and/or its mRNA or pre-mRNA transcript. They include antisense RNA molecules, RNAi molecules and ribozymes. See, e.g. ref. 16 for examples of RNAi and other molecules which have been expressed under the control of pol III promoters.

Antisense oligonucleotides hybridise with complementary sequences of RNA generally by Watson-Crick base pairing. The resultant double stranded complex prevents translation of the message into protein product either by steric blocking at the ribosome or activation of RNase H that cleaves the RNA strand of the duplex. Molecules capable of binding to the translation initiation site of the coding DNA , e.g. between the -10 and +10 regions of the target gene, may also be used.

The complete sequence corresponding to the coding sequence need not be used. For example fragments of sufficient length may be used. It is a routine matter for the person skilled in the art to screen fragments of various sizes and from various parts of the coding sequence to optimise the level of antisense

inhibition. It may be advantageous to include the initiating methionine ATG codon, and perhaps one or more nucleotides upstream of the initiating codon. A further possibility is to target a conserved sequence of a gene, e.g. a sequence that is characteristic of one or more genes, such as a regulatory sequence.

The sequence employed may be 500 nucleotides or less, possibly about 400 nucleotides, about 300 nucleotides, about 200 nucleotides, or about 100 nucleotides. It may be possible to use oligonucleotides of much shorter lengths, 14-23 nucleotides, although longer fragments, and generally even longer than 500 nucleotides are preferable where possible.

It may be preferable that there is complete sequence identity in the sequence used for down-regulation of expression of a particular gene, and the gene to be down-regulated. However total complementarity or similarity of sequence is not essential. One or more nucleotides may differ in the sequences used. Thus, a sequence employed in a down-regulation of gene expression in accordance with the present invention may be a wild-type sequence (e.g. gene) selected from those available, or a mutant, derivative, variant or allele, by way of insertion, addition, deletion or substitution of one or more nucleotides, of such a sequence. The sequence need not include an open reading frame or specify an RNA that would be translatable. It may be preferred for there to be sufficient homology for the respective anti-sense and sense RNA molecules to hybridise. There may be down regulation of gene expression even where there is about 5%, 10%, 15% or 20% or more mismatch between the sequence used and the gene whose expression is to be down-regulated.

Double stranded RNA (dsRNA) has been found to be even more effective in gene silencing than antisense strands alone (Fire A. et al Nature, Vol 391, (1998)). dsRNA mediated silencing is gene specific and is often termed RNA interference (RNAi). RNA interference is a two step process. First, dsRNA is cleaved

within the cell to yield short interfering RNAs (siRNAs) of about 21-23nt length with 5' terminal phosphate and 3' short overhangs (~2nt) The siRNAs target the corresponding mRNA sequence specifically for destruction (Zamore P.D. Nature Structural Biology, 8, 9, 746-750, (2001). See also Fire (1999) Trends Genet. 15: 358-363, Sharp (2001) Genes Dev. 15: 485-490, Hammond et al. (2001) Nature Rev. Genes 2: 1110-1119 and Tuschl (2001) Chem. Biochem. 2: 239-245. In mammalian cells, due to the undesirable induction of the interferon response with long dsRNA molecules, use of dsRNA molecules of less than 30 nucleotides is usually required. To this end, constructs which express single stranded RNAs which would form "hair-pin-loop" structures due to the presence of an inverted repeat, the hairpin part usually capable of forming a dsRNA segment of 21 nucleotides (such as that shown in figure 4) are preferentially used. The single stranded RNA ends and the loop are effectively removed in vivo to leave functional siRNAs.

By way of example, Figure 4 illustrates a construct based on p670 (Figure 1) which uses the EBV EBER 1 promoter, supplemented by a pol III Eμ enhancer, to express a siRNA targeting EBER 1. This construct will direct expression to lymphoid cells.

Ribozymes are enzymatic RNA molecules capable of catalysing the specific cleavage of RNA. (For a review, see Rossi, J., 1994, Current Biology 4: 469-471). The mechanism of ribozyme action involves sequence specific hybridisation of the ribozyme molecule to complementary target RNA, followed by an endonucleolytic cleavage. The composition of ribozyme molecules must include one or more sequences complementary to the target protein mRNA, and must include the well known catalytic sequence responsible for mRNA cleavage. For this sequence, see US Pat. No. 5,093,246, which is incorporated by reference herein in its entirety. As such, within the scope of the invention are engineered hammerhead motif ribozyme molecules that specifically and efficiently

catalyse endonucleolytic cleavage of RNA sequences encoding target proteins.

Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the molecule of interest for ribozyme cleavage sites which include the following sequences, GUA, GUU and GUC. Once identified, short sequences of between 15 and 20 ribonucleotides corresponding to the region of the target protein gene containing the cleavage site may be evaluated for predicted structural features, such as secondary structure, that may render the oligonucleotide sequence unsuitable. The suitability of candidate sequences may also be evaluated by testing their accessibility to hybridise with complementary oligonucleotides, using ribonuclease protection assays.

The constructs may be used to create transgenic animals. Thus a nucleic acid construct according to the invention may be introduced into the genomic DNA of the animal in order to obtain an animal which displays tissue-specific, pol III-driven expression of the target sequence. This raises the possibility of creating animals in which expression of desired genes is inhibited in a tissue-specific manner, by use of target sequences encoding appropriate antisense, RNAi or ribozyme molecules.

Alternatively, a suitable pol II regulatory element may be introduced (e.g. by homologous recombination) such that it becomes operably linked to a pol III promoter already present in the genomic DNA of the subject in order to create a construct as described herein. Such methods are considered to be within the scope of the invention.

Use of the constructs is not limited to inhibition of gene expression. It may be desirable to express pol III transcripts for other purposes. For example, pol III transcripts which mimic elements of the HIV genome have been proposed for therapeutic use. These molecules would serve as "decoy" RNAs, competing for

binding proteins and so preventing the proteins from binding the viral genome to perform their function in viral replication.

This technology may be applied as desired to any appropriate non-human animal subject, whether vertebrate or invertebrate, including trypanosomes, insects (e.g. Drosophila), worms (e.g. Caenorhabditis elegans), fish (e.g. zebrafish), reptiles, amphibians (e.g., frogs and toads, especially Xenopus laevis) and mammals, including rodents (mice, rats, guinea pigs, hamsters) lagomorphs (e.g. rabbits), domestic and agricultural animals (e.g. dogs, cats, horses, sheep, goats, cows, and primates including monkeys (e.g., marmoset, baboon) and apes (e.g., gorilla, chimpanzee, orangutang, gibbon).

Of course, it will be appreciated that human cells may be manipulated in vitro using the constructs and methods described herein, and that humans may be treated using the therapeutic methods described.

Pharmaceutical compositions

The constructs of the invention may find use in methods of therapy, and so may be formulated as pharmaceutical compositions.

These compositions may comprise, in addition to one of the above substances, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material may depend on the route of administration, e.g. oral, intravenous, cutaneous or subcutaneous, nasal, intramuscular, intraperitoneal routes or topical application.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant. Liquid

pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

Administration is preferably in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors, and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners. Suitable carriers, adjuvants, excipients, etc. can be found in standard pharmaceutical texts, for example Remington's Pharmaceutical Sciences, 20th Edition, 2000, pub. Lippincott, Williams & Wilkins; and Handbook of Pharmaceutical Excipients, 2nd edition, 1994.

The constructs of the invention may be used in methods of gene therapy, and may be introduced into cells of a recipient by any suitable means, such that the target sequence is transcribed by pol III in the desired cells.

The construct may be introduced in the form of naked DNA, which is taken up by some cells of animal subjects, including muscle cells of mammals. In this aspect of the invention the construct will generally be carried by a pharmaceutically acceptable carrier alone. The construct may also encapsulated, e.g. by formulation in an inert polymer or a liposome particle.

Such methods of gene therapy further include the use of recombinant viral vectors such as adenoviral or retroviral vectors which comprise a construct of the invention. Such viral vectors may be delivered to the body in the form of packaged viral particles. The viral vectors may themselves be targeted to the appropriate cells.

Gene therapy methods are widely documented in the art and may be adapted for use in the expression of the required sequence.

EXAMPLES

Experimental procedures:

1. Plasmid and transgene construction:

For the different EBER1 constructs 4 PCR primers were designed incorporating either an *Eco*RI or an *Xho*I restriction site (shown in the table below, the restriction sites are underlined):

Primer name	5′ to 3′ primer sequence
CR1	gtgtgtgtgaattcgtcagcctgcaaggtg
CR2	gtgtgtgtgaattcactatagcaaaccccg
CR3	gtgtgtgtgaattctcttgaggagatgtag
CR4	gtgtgtgtctcgagaaaagatgcggaccac

The PCR reactions were performed on pLexIII plasmid containing EBER1 sequence (gift from Dr Mike Clemens) with *Pfu* polymerase enzyme. The cycling conditions are shown in the table below:

Temperature	Time	Number of cycles
95°C	5 minutes	1
95°C	30 seconds	30
60°C	30 seconds	
72°C	1 minute	
72°C	10 minutes	1

Three different PCR reactions were performed in order to generate the three different EBER1 constructs using primers: CR1-CR4, CR2-CR4, CR3-CR4. The first EBER1 construct contains the upstream sequences of EBER1 (to -322) and EBER1 (called p670, using CR1 and CR4 primers). The second has only the upstream elements required for EBER1 expression (Sp1, ATF and the TGTA box) and EBER1 (called p671, using CR2 and CR4 primers) and the third construct has only the TGTA box of EBER1 and EBER1 sequence (called p672, using CR3 and CR4 primers) (see figure 1).

The PCR products were digested with *Eco*RI and *Xho*I and then ligated into pcDNA3.1A, which was linearised with *Eco*RI and *Xho*I enzymes (other commercially available vectors could equally be used as the plasmid backbone). The new plasmids were transformed into DH5 cells. After performing a minipreparation of plasmid DNA from several colonies the plasmids with the correct restriction pattern were sequenced to confirm that the correct product had been obtained. An EndoFree preparation of the plasmid DNA was performed according to the manufacturer's instructions (Qiagen).
*Xba*I linear fragments were used as transgenes for microinjection of zygotes at 2 and 5µg/ml.

2. Pronuclear microinjection of B6D2.F1 mouse embryos:

48 and 24 hours prior to microinjections B6D2.F1 mice were injected with 5 units of pregnant mare's serum gonadotropin (PMS) and chorionic gonadotropin (hCG) respectively. The mice were set up with B6D2.F1 stud males the day before microinjections. Embryos were harvested and placed in M2 medium (Sigma) and at 37°C, 5% CO_2. For the pronuclear microinjections the embryos were transferred to a depression slide in M16 medium (Sigma). Successfully microinjected embryos were implanted into pseudopregnant ICR females. Founders were backcrossed to the C57Bl/6 strain to generate transgenic mouse lines.

3. Genomic DNA analysis of the different mice generated:

Mouse tail DNA was isolated by digestion with proteinase K and tail solution at 55°C shaking overnight and the genomic DNA was purified according to the protocol described by Wilson et al. (12, 13). The DNA was then digested with the appropriate restriction enzyme and electrophoresed through an agarose gel. This was followed by Southern blotting using the correct probe as described (12, 13).

4. RNA extraction, Northern blots and reverse-transcriptase (RT)-PCR:

Total RNA was isolated from tissues or cell pellets using the method described by Chomczynski and Sacchi (2). 20µg of total RNA was used for Northern blotting using the protocol described by Wilson *et al.*, (12, 13). Reverse-transcriptase reactions were performed with 2µg of total RNA using an EBER1 specific primer (CR4) and M.MLV enzyme (Invitrogen). A control was performed for each sample without any RT enzyme. The PCR was performed using EBER1 primers and 1/5th of the RT reaction as template. The primer sequence is shown in the following table:

Primer name	5′ to 3′ primer sequence
CR8	aggacctacgctgc

CR9	tacttgaccgaagac

The cycling conditions are shown in the table below:

Temperature	Time	Number of cycles
95°C	5 minutes	1
95°C	30 seconds	25
40°C	30 seconds	
72°C	30 seconds	
72°C	10 minutes	1

The RT-PCR products were electrophoresed on a 2% agarose gel and generally followed by Southern blotting using an EBER1 probe.

5. Quantitative-RT-PCR (QPCR):

Quantitative PCR was usually performed following an RT reaction. The SYBRGreen kit from ABgene was used and QPCR was performed using CR8 and CR9 EBER1 primers and 1/5th of the RT reaction as template. The cycling conditions were the same as the ones described in 4 except for a hot start of 15 minutes instead of 5 and a plate read analysis of the samples after each cycle. A standard curve was also performed alongside the samples. Each sample for the standard curve had a known quantity of EBER1 template. From the standard curve equation the initial quantity of cDNA in each sample could be calculated. The reactions were performed on the DNA engine Opticon™ from MJ Research and the results analysed using Opticon programme.

6. Tissue culture and transient transfections:

The murine B cell line used for transient transfections of the different EBER1 constructs was generated in the lab from an EBV latent gene-positive tumour and is referred to as 39.415. The cells were grown in RPMI medium complemented with 10% FCS, 2% penicillin/streptomycin and 2% l-glutamine (Gibco). The cells

were incubated at 37°C and 5% CO_2. They were passaged every 2 to 3 days.

For the transient transfections 10^7 cells were centrifuged and resuspended in 250µl of serum free medium (SFM). 5µg of DNA was used for the transfections and added to a final volume of 50µl of SFM and placed in a 4mm cuvette (CLP). The cells were then added and mixed to the DNA in the cuvette, which was placed on ice for 5 minutes. Electroporation was performed using the Gene Pulser® electroporation apparatus (Biorad). The parameters used for the transfection were 250V and 960µF. Following electroporation the cells were placed at 37°C for 10 minutes and then added to 10ml of medium. The cells were either harvested at 24 or 48 hours post transfection.

Results:

1. Design of the vector:

Three different EBER1 constructs were designed to test if spacing between the elements is critical and if the SP1 and ATF sites are essential for efficient expression. The vector backbone used was pcDNA3.1A (Invitrogen). The immunoglobulin heavy chain (IgH) enhancer Eµ enhancer (17) was cloned upstream of the three different EBER1 constructs (see figure 1) as described in experimental procedures (section 1) to direct the expression to the B cell compartment. This enhancer has been previously used in the laboratory to direct the expression of pol II transcripts encoding several heterologous genes to the B-cell compartment (1, 11, 13). The TGTA box of EBER1 was maintained in all constructs as it has been shown that this element is important for EBER2 transcription by RNA pol III (6) and therefore may also be critical for EBER1 expression.

The plasmids containing the different EBER1 constructs were sequenced before the preparation of the transgene to check that no mutations had been generated during construction. The

transgene was prepared using endotoxin free plasmid DNA preparations so as not to trigger an immune response in tissue culture assays or *in vivo*, which could obscure any effect due to EBER1 expression.

2. Expression in culture of the three different EBER1 vectors:

The efficacy of expression of each construct was tested in culture. The different constructs were transiently transfected into EBER-negative B cells. Following RNA extraction the level of expression of EBER1 in the three different constructs was determined by reverse transcriptase PCR (RT-PCR), quantitative-RT-PCR (QPCR) and Northern blots. All three assays showed expression of the different constructs in culture (figure 2).

3. Generation of EBER1 transgenic mice and expression:

Pronuclear microinjections were performed using B6D2.F1 mouse embryos and the surviving embryos were implanted in pseudopregnant ICR females. 6 founders for transgene 670, 3 founders for 671 and 10 founders for 672 were generated. Several of the founders have been bred to generate lines which are currently under investigation for expression analysis of the transgene and phenotype. Initially, 2 of the lines carrying the p671 transgene were checked for expression of the transgene. RNA extraction from collected tissues from transgenic positive mice and negative siblings was followed by RT-PCR. The expression in the lines is specific to the lymphoid tissues and the brain (figure 3) and no other tissues tested (liver, muscle, ovaries, uterus, testis, kidneys, small intestine, stomach, heart, lung, trachea, oesophagus, tongue, nasopharyngeal region, dorsal skin, ears, salivary gland). This demonstrates that the combination of pol II and pol III elements is functional and that the pol II Eμ enhancer is compatible with pol III elements. This is the first report demonstrating the achievement of tissue specific pol III expression.

Two of three lines carrying p670 and 6 of 8 lines carrying p672 have also been shown to express their respective transgenes. The majority of these lines show lymphoid-specific expression as exemplified by Figure 3. Some variation in expression is to be expected as the transgenes integrate into the mouse genome at random locations. As a result, they may become integrated at "silent sites" from which expression is not possible, or may adventitiously become integrated under the control of chromosomal sequences which affect transgene expression.

Discussion

RNA pol III genes are abundantly and ubiquitously expressed. Therefore use of these promoters with heterologous genes in transgenic animals or in DNA vaccination may well be lethal or undesirable. Several experimental approaches might require a targeted expression of the gene of interest. This is especially true in therapeutic protocols. Therefore we have developed a vector combining pol II and pol III elements to express a pol III gene in a tissue specific manner. In a proof of concept we have used this vector to express EBER1 in B cells in culture and in the lymphoid tissues of transgenic mice.

A different pol II tissue specific enhancer element could be used, simply by switching the Eμ motif with another in order to direct the expression of the gene of interest to a different tissue. A different gene could be used in the vector; for instance, other pol III genes with A and B boxes could readily replace the EBER1 sequence.

The vectors described here may be used for numerous application including RNA interference (RNAi). RNAi is a process through which exposure of dsRNA leads to the silencing of the homologous gene, most often post-transcriptionally (4). RNA silencing was first observed in plants and this phenomenon was termed post-transcriptional gene silencing (PTSG). RNAi is a widespread phenomenon which has been intensively used to characterise the

functions of genes in *Caenorhabditis elegans*, *Drosophila* and trypanosomes. RNAi can be performed in mammalian cells using 21-nucleotide siRNA duplexes which do not induce an interferon response (3). RNAi transgenes expressing short hairpin RNA could be used as an alternative to generating mouse knock-out (or more accurately, "knock-down") models. Here again, tissue specificity would be desirable. RNAi has enormous therapeutic potential and could be used *in vivo* to silence disease or other deleterious genes. For instance, the siRNA sequence of the target gene could be inserted before and after the B box of EBER1 using the B box sequence as the loop of the "hairpin".

While the invention has been described in conjunction with the exemplary embodiments described above, many equivalent modifications and variations will be apparent to those skilled in the art when given this disclosure. Accordingly, the exemplary embodiments of the invention set forth are considered to be illustrative and not limiting. Various changes to the described embodiments may be made without departing from the spirit and scope of the invention. All references cited herein are expressly incorporated by reference.

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Claims:

1. A nucleic acid molecule comprising an expression cassette, the expression cassette comprising a target sequence to be transcribed by RNA pol III operably linked to RNA pol III transcriptional regulatory elements, said expression cassette further comprising a pol II transcriptional regulatory element which provides selective transcription of genes by RNA pol II in a target cell or tissue, whereby said target sequence is selectively transcribed by RNA pol III in said target cell or tissue.

2. A nucleic acid molecule according to claim 1 wherein the pol II transcriptional regulatory element is a pol II enhancer element.

3. A nucleic acid molecule according to claim 2 wherein the enhancer binds to a transcription factor capable of stimulating transcription of the target sequence by pol III, and wherein the transcription factor is endogenous to the target cell or tissue.

4. A nucleic acid molecule according to claim 2 or claim 3 wherein the enhancer provides lymphoid specific expression.

5. A nucleic acid molecule according to claim 4 wherein the enhancer is an immunoglobulin Eμ enhancer.

6. A nucleic acid molecule according to any one of claims 1 to 5 wherein the RNA pol III transcriptional regulatory elements comprise an A block and either a B block or C block located downstream of the transcriptional start site.

7. A nucleic acid molecule according to claim 6 wherein the pol III transcriptional regulatory elements may further comprise one or more upstream elements.

8. A nucleic acid molecule according to claim 7 wherein the upstream elements comprise a TATA-like box

9. A nucleic acid molecule according to any one of claims 1 to 5 wherein the pol III regulatory elements may consist entirely of upstream elements.

10. A nucleic acid molecule according to claim 9 wherein the pol III regulatory elements comprise a TATA-like box.

11. A nucleic acid molecule according to any one of claims 1 to 5 wherein the pol III regulatory elements comprise some or all of the elements of an EBV EBER promoter.

12. A nucleic acid molecule according to claim 11 wherein the elements of the EBER promoter comprise the A and B blocks located downstream of the initiation site.

13. A nucleic acid molecule according to claim 11 or claim 12 wherein the elements of the EBER promoter comprise the TATA-like box located upstream of the initiation site.

14. A nucleic acid molecule according to any one of claims 1 to 13 wherein the target sequence encodes a RNA molecule capable of inhibiting the expression of a target gene in the same cell.

15. A nucleic acid molecule according to claim 14 wherein the encoded RNA molecule is an antisense RNA molecule, RNAi molecule, siRNA molecule or ribozyme.

16. A nucleic acid molecule according to claim 14 or claim 15 wherein the encoded RNA molecule is capable of inhibiting EBER expression in an Epstein Barr Virus (EBV)-infected cell and is expressed specifically in B cells.

17. Use of a nucleic acid molecule according to claim 17 in the manufacture of a medicament for the treatment of a condition associated with EBV infection.

18. Use according to claim 17 wherein the condition is a tumour.

19. Use according to claim 18 wherein the tumour is Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's disease or an AIDS related B-cell lymphoma.

20. A nucleic acid molecule according to any one of claims 1 to 16 for use in a method of medical treatment.

21. An expression vector comprising a nucleic acid molecule according to any one of claims 1 to 16.

22. A cell comprising a nucleic acid according to any one of claims 1 to 16, or an expression vector according to claim 21.

23. A non-human transgenic animal comprising a nucleic acid according to any one of claims 1 to 16 or an expression vector according to claim 21.

24. A method of modulating transcription of a target sequence operably linked to RNA pol III transcriptional regulatory elements, the method comprising operably linking said target sequence to a pol II transcriptional regulatory element which provides selective transcription of genes by RNA pol II in a target cell or tissue, whereby said target sequence is selectively transcribed by RNA pol III in said target cell or tissue.

25. A method according to claim 24 wherein said target sequence is operably linked to said pol II transcriptional regulatory element by introducing said pol II regulatory element into the chromosome of a cell.

26. A method according to claim 25 wherein said introduction is performed by homologous recombination.

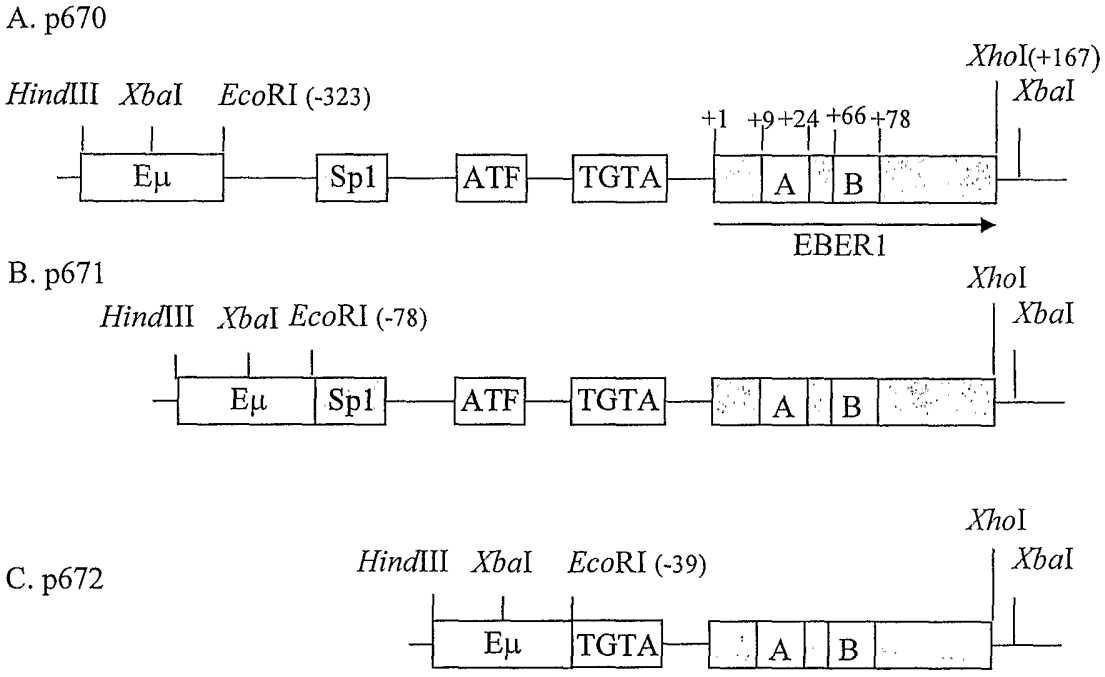

A. p670
HindIII XbaI EcoRI (-323)
XhoI(+167)
XbaI
+1 +9 +24 +66 +78
Eμ
Sp1
ATF
TGTA
A
B
EBER1
B. p671
HindIII XbaI EcoRI (-78)
XhoI
XbaI
Eμ
Sp1
ATF
TGTA
A
B
C. p672
HindIII XbaI EcoRI (-39)
XhoI
XbaI
Eμ
TGTA
A
B

Figure 1

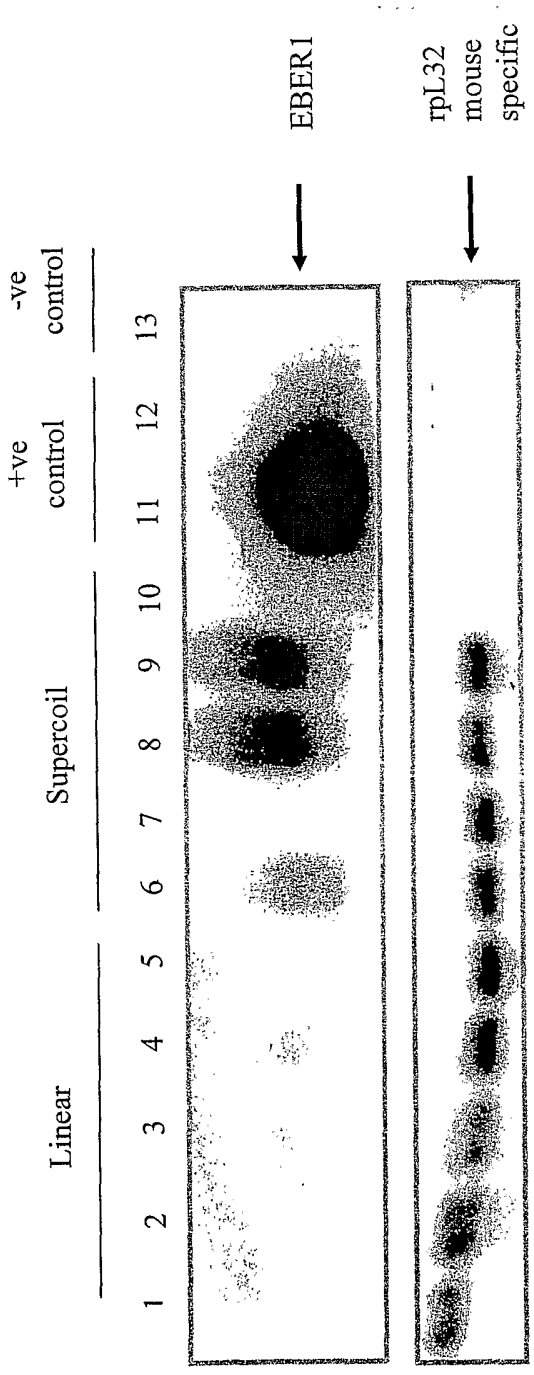

EBER1
rpL32 mouse specific
Linear
Supercoil
+ve control
-ve control
1 2 3 4 5 6 7 8 9 10 11 12 13

Figure 2

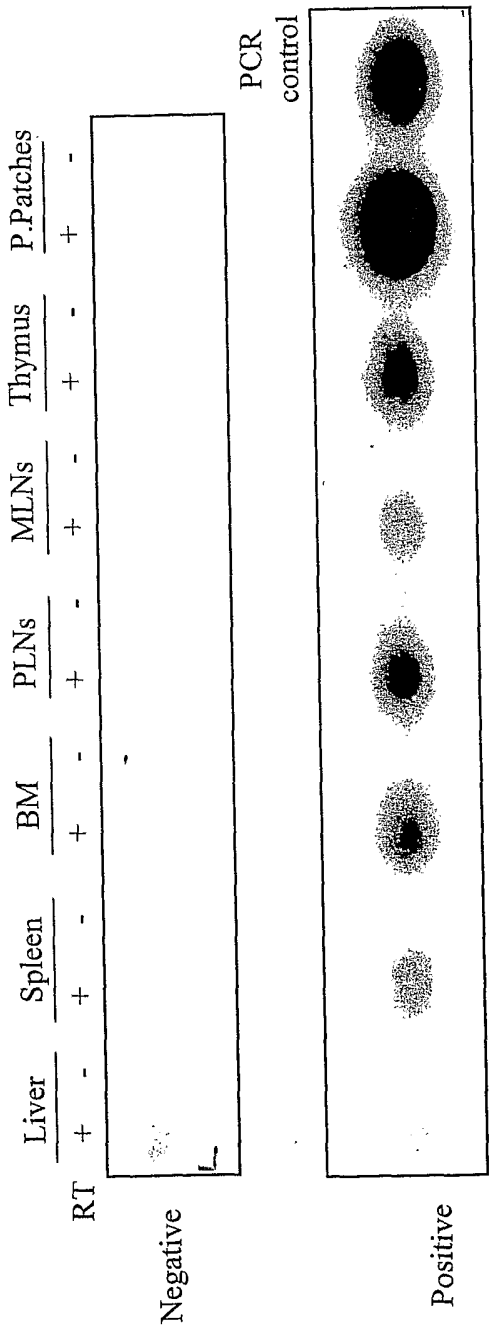
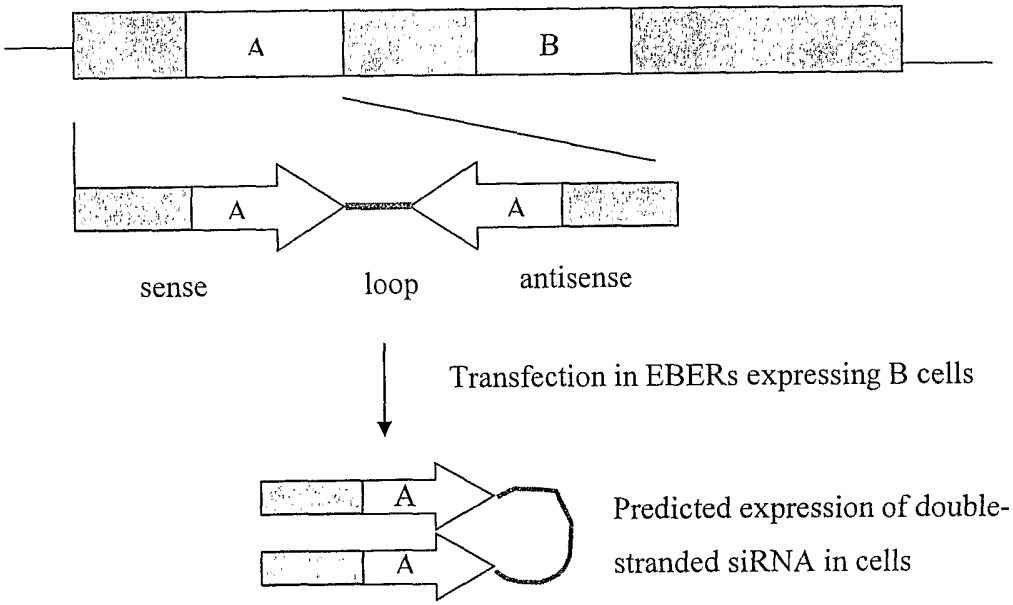

RT
Liver
Spleen
BM
PLNs
MLNs
Thymus
P.Patches
+ - + - + - + - + - + -
Negative
PCR control
Positive

Figure 3

A



B

gaattcgtcagcctgcaaggtggatggcgtgttttctgaggttatccccgctacgtg
catgctgggtgatagagaccctagaatgtgtcgaaatgaccaagcgtccccgcagcg
gggctcccaacacgggttcccagagagggtaaaagaggggccataaagcccagggt
gtaaaacaccgaccgcgccaccagatggcacacgtgggggaaatgagggttagcata
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CTAGAGGTTCAAGAGACCTCTAGGGCAGCGTAGGCTTTTTTTgctagggaggagacg
tgtgtggctgtagccacccgtccc***gggtacaagtccc***gggtggtgaggacggtgtct
gtggttgtcttcccagactctgctttctgccgtcttcggtcaagtaccagctggtgg
tccgcatgttttgagctc

Figure 4

C

AGCAGCTGGC AGGAAGCAGG TCATGTGGCA AGGCTATTTG GGGAAGGGAA
AATAAAACCA CTAGGTAAAC TTGTAGCTGT GGTTTGAAGA AGTGGTTTTG
AAACACTCTG TCCAGCCCCA CCAAACCGAA AGTCCAGGCT GAGCAAAACA
CCACCTGGGT AATTTGCATT TCTAAAATAA GTTGAGGATT CAGCCGAAAC
TGGAGAGGTC CTCTTTTAAC TTATTGAGTT CAACCTTTTA ATTTTAGCTT
GAGTAGTTCT AGTTTCCCCA AACTTAAGTT TATCGACTTC TAAAATGTAT TTAG

Figure 4 (contd.)

INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/GB2005/003599

A. CLASSIFICATION OF SUBJECT MATTER
C12Q1/68 C12N15/67

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q C12N

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, EMBASE, FSTA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHESNOKOV I ET AL: "Flanking sequences of an Alu source stimulate transcription in vitro by interacting with sequence-specific transcription factors." JOURNAL OF MOLECULAR EVOLUTION. JAN 1996, vol. 42, no. 1, January 1996 (1996-01), pages 30-36, XP009056110 ISSN: 0022-2844 the whole document ----- -/--	1,20-22, 24

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
2 November 2005	22/11/2005

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Leber, T

INTERNATIONAL SEARCH REPORT

Inte ıal Application No
PC1/GB2005/003599

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A	GABRIELSEN O S ET AL: "RNA polymerase III (C) and its transcription factors." TRENDS IN BIOCHEMICAL SCIENCES. NOV 1991, vol. 16, no. 11, November 1991 (1991-11), pages 412-416, XP002352131 ISSN: 0968-0004 the whole document	

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