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(54) Title: METHOD OF INHIBITING VIRAL PRODUCTION

(57) Abstract

The present invention relates to a method of treating a viral infection utilizing antisense oligonucleotides that specifically inhibit expression of genes involved in virus replication. The invention also relates to antisense oligomers suitable for use in such a method and to pharmaceutical compositions comprising same.

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+ Any designation of "SU" has effect in the Russian Federation. It is not yet known whether any such designation has effect in other States of the former Soviet Union.

METHOD OF INHIBITING VIRAL PRODUCTION

BACKGROUND OF THE INVENTION

Technical Field

The present invention relates, in general, to a method of treating viral infection, and, in particular, to a method of treating Epstein-Barr virus (EBV) associated diseases. The invention further relates to oligomers (oligonucleotides) suitable for use in such a method, and to pharmaceutical compositions comprising same.

Background Information

Viruses produce a series of antigens upon infection of a given cell. These antigens can be subdivided into two major categories, latent and lytic. Latent antigens are viral antigens not directly associated with the replication cycle of the virus, but in the case of EBV, are required for the maintenance of the viral genome within the infected cell.

Lytic antigens are antigens directly associated with virus replication, and can be further subdivided into early versus late. Early antigens differ from late in that they are expressed early in the viral replication cycle, and require no new viral DNA synthesis for their expression. These early antigens (EA) most likely function in the early stages of viral DNA replication, although the specific function of each component of this group remains unknown.

The major restricted early antigen of EBV (EA-R) has been found to be a viral specific ribonucleotide reductase (Goldschmidts et al, Virology 157:220-226 (1987)). This enzyme is responsible for mediating the first unique step of DNA synthesis by reducing all four

ribonucleotides to their corresponding deoxyribonucleotides.

One skilled in the art will appreciate that methods of reducing the production of specific viral antigens might well be used in the treatment of viral-associated diseases. The present invention provides such methods and oligonucleotides suitable for use in same.

SUMMARY OF THE INVENTION

It is a general object of the invention present invention to provide anti-sense oligonucleotides that inhibit lytic and latent viral gene expression.

It is a specific object of the invention to provide a method of combatting EBV associated diseases and oligonucleotides suitable for use therein.

Further objects and advantages of the present invention will be apparent from the description of the invention that follows.

In one embodiment, the present invention relates to an antisense oligonucleotide that selectively inhibits expression of a viral antigen.

In another embodiment, the present invention relates to a method of inhibiting expression of a viral antigen. The method comprises contacting the virus with the above-described oligonucleotide under conditions such that inhibition of expression is effected.

In a further embodiment, the present invention relates to a method of treating viral infection. The method comprises contacting a virally infected cell with the above-described oligonucleotide under conditions such that the treatment is effected.

In yet another embodiment, the present invention relates to a pharmaceutical composition, in dosage unit

form. The composition comprises the above-described oligonucleotide in an amount sufficient to effect the selective inhibition, together with a pharmaceutically acceptable carrier.

5 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: BamH1 map of B95-8 strain of EBV.

Figure 2: Prevention of progression of EBV into the lytic cycle by treatment of EBV infected cells with anti-EA-R oligonucleotides.

10 Figure 3: Sequence specific inhibition of EA-R and EA-D antigens of EBV within dosage and time window.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a method of treating a viral infection utilizing antisense 15 oligonucleotides that specifically inhibit expression of genes involved in virus replication. The invention also relates to antisense oligomers suitable for use in such a method and to pharmaceutical compositions comprising same.

20 The antisense oligonucleotides of the invention are designed so as to be sufficiently complementary to a specific viral messenger RNA to form a stable hybrid therewith (see in this regard Heikkila et al, Nature 7:445 (1987); Holt et al, Mol. Cell. Biol. 8:963 25 (1988); see also McManaway et al, Lancet 335:808-811 (1990)). Advantageously, the oligonucleotides target lytic antigens, preferably, early lytic antigens.

In one embodiment, the oligonucleotides of the invention are specific for the EA-R antigen of EBV. 30 One example of such an oligonucleotide has the sequence 3' TGG-TGT-GTC-GTA-CCG-TTG-CTG 5' (see Figure 1).

Oligonucleotides suitable for use in the present invention can be selected by choosing accessible

regions in the messenger RNA, specifically regions flanking the start codon. Other regions including the CAP site may also be useful. The oligomers of the invention can be synthesized using methods known in the
5 art (Schott, M.E. Am. Biotech. Lab. 3:20-23 (1985)).

The oligonucleotides of the invention can be formulated together with an appropriate carrier, diluent or excipient, into a pharmaceutical composition. Such compositions can be administered
10 intraperitoneally, intravenously, intrathecally or by injection at the site of the lesion. The concentration of oligonucleotide present in the composition is, for example, about 100 μM to about 1 mM. Advantageously a similar concentration level is achieved at the lesion
15 site. Actual concentrations can be adjusted so as to be applicable to the particular disease state. Optimum doses can be determined using methods known in the art.

In one embodiment, the pharmaceutical composition of the invention is formulated so as to be suitable for
20 topical application. Topical application of oligomers obviates oligonucleotide degradation which can be a problem with parenterally administered oligonucleotides. Further, topical application ensures adequate concentrations of oligomer at the target site.
25 The oligonucleotides of the invention can be used in methods of treating viral infection, including EBV infection and in treating viral associated tumors. Application of the oligomers to either treatment methodology can have a variety of effects, including
30 obliteration of virus replication within infected cells. In the case of Hairy Leukoplasia, for example, which illness is associated with EBV replication in cells of the tongue and oral cavity, the application of

the antisense oligonucleotides of the invention is expected to prevent the spread of the virus to other cells and thereby control the disease. This treatment can be achieved by local or topical application of the
5 oligonucleotide in a form suitable for such application.

In illnesses such as Burkitt's lymphoma, nasopharyngeal carcinoma, and lymphoproliferative syndromes associated with immunosuppression (including
10 HIV associated lymphomas), where virus replication is not only not associated with pathogenesis, but would cause cell lysis and disease regression, the expression of so called EBV "latent" genes such as EBNA-1 and LMP may be linked to the maintenance of the neoplastic
15 state. In these cases, use of antisense oligonucleotides directed toward these specific EBV genes is contemplated. Such use would be expected to cause tumor regression. The viral genes can be expected to be inhibitable with impunity, since they
20 are foreign to the cell and, therefore, not necessary for any normal cellular function.

The method of the present invention should not result in impairment of immunity against the viral infection, unless it results in elimination of all EBV
25 containing cells from the body (a possibility if the gene which is inhibited by antisense is responsible for maintenance of the latent infection, i.e. EBNA-1). However, where this is not the case, cells are likely to continue to express other viral genes responsible
30 for immunity. If elimination of infection were to occur, however, the present method would be of great value in patients being prepared for bone marrow

transplantation, since EBV lymphoproliferative states may occur in such individuals.

Antisense oligomers can also be applied to other conditions shown to be due, at least in part, to expression of viral genes. This includes other malignant conditions, e.g. peripheral T cell lymphomas and leukemias associated with HTLV-1 virus (inhibition of tat, responsible for IL-2 and IL-2 receptor induction). It is likely that viruses not yet discovered also participate in the pathogenesis of malignancy. Infectious conditions, in particular, CMV, and herpes (simplex and zoster) infections, scourges of bone marrow transplantation and immunodeficiencies, such as AIDS, can also be expected to respond to such treatment.

The following examples illustrate certain aspects of the invention in greater detail.

Example 1

Inhibition of EA-R by Sequence Specific Oligonucleotide

P3HR-1, an EBV genome positive Burkitt's lymphoma cell line, was chemically stimulated to produce lytic viral antigens by the addition of 3 mM sodium butyrate and 20 ng of tumor-promoting agent (TPA) per milliliter. One hour prior to the chemical induction and two hours after, antisense and sense (control) oligonucleotides were added to individual wells at a final concentration of 200μM, and incubated for 30 hours (see Figure 1 for oligomer sequences).

After determining cell viability, equal numbers of cells were harvested, extracted, and exposed to SDS-PAGE. The gels were subsequently transferred to nitrocellulose paper, and then incubated with

appropriate antibodies directed to the various viral antigens of interest.

The decreased expression of EA-R observed in the cells was accompanied by a parallel decrease in the production of a 50-52 kDa diffuse early antigen (EA-D), suggesting the expression of the two early antigens may be coupled. Inhibition of these early antigens dramatically inhibited the progression of the induced cells to virus production, as monitored by the expression of viral capsid proteins (antigens) (VCA). These results indicate that the lytic cycle of EBV can be efficiently aborted by use of antisense oligonucleotides directed toward the restricted early antigen of EBV.

The results are shown in Figure 2:

Lane A- Cells exposed to sense (control) oligonucleotides, and stained with a mixture of monoclonal antibodies directed to EA-R and EA-D antigens of EBV; cells show no inhibition of either antigen.

Lane B- Cells exposed to sense (control) oligonucleotides, and stained with a polyclonal rabbit serum directed to the major viral capsid antigen (VCA) of EBV; cells show no inhibition of VCA.

Lane C- Cells exposed to EA-R specific oligonucleotides, and stained with a mixture of monoclonal antibodies directed to EA-R and EA-D antigens of EBV; cells show inhibition of both antigens.

Lane D- Cells exposed to EA-R specific oligonucleotides and stained with a polyclonal rabbit serum directed to the major viral capsid antigen (VCA)

of EBV; cells show inhibition of VCA. (note background bands stay the same).

Example 2

In Inhibition of EA-R with Dosage and Time

5 Individual wells of induced P3HR-1 cells were expose at various time points to a final concentration of 300 μM antisense or nonsense oligonucleotides (see Figure 1), and harvested 30 hours after induction of the viral lytic cycle. After determining cell 10 viability, equal numbers of cells were harvested, extracted, and exposed to SDS-PAGE. The gels were subsequently transferred to nitrocellulose paper, and then incubated with a mixture of monoclonal antibodies directed toward the EA-R and EA-D antigenes of EBV.

15 The results are shown in Figure 3:

A. Western blot of P3HR-1 cells stained with a mixture of monoclonal antibodies directed against EA-R and EA-D antigens.

B. Western blot of corresponding cell lysates shown in 20 Panel A, stained with a monoclonal antibody against vimentin (control cellular protein).

\+ Induced cells without addition of oligonucleotides.

\- Uninduced cells without addition of 25 oligonucleotides.

Lane 1 Induced cells which received antisense oligonucleotides one hour prior to and two hours post induction.

Lane 2 Induced cells which received nonsense 30 oligonucleotides one hour prior to and two hours post induction.

Lane 3 Induced cells which received antisense oligonucleotides one hour prior to induction.

Lane 4 Induced cells which received nonsense oligonucleotides one hour prior to induction.

5 Lane 5 Induced cells which received antisense oligonucleotides two hours post induction.

Lane 6 Induced cells which received nonsense oligonucleotides two hours post induction.

Lane 7 Induced cells which received antisense
10 oligonucleotides twelve hours post induction.

Lane 8 Induced cells which received nonsense oligonucleotides twelve hours post induction.

* * * *

For purposes of completing/supplementing this
15 disclosure, the entire contents of all references cited hereinabove are incorporated by reference.

The foregoing invention has been described in some detail for purposes of clarity and understanding. One skilled in the art will appreciate, however, that
20 various changes in form and detail can be made without departing from the true scope of the invention.

WHAT IS CLAIMED IS:

1. An antisense oligonucleotide that selectively inhibits expression of a viral antigen.

2. The oligonucleotide according to claim 1 wherein said oligonucleotide selectively hybridizes to messenger RNA of said antigen in a manner such that expression of said antigen is inhibited.

3. The oligonucleotide according to claim 1 wherein said antigen is a lytic antigen.

4. The oligonucleotide according to claim 3 wherein said antigen is an early lytic antigen of Epstein-Barr virus (EBV).

5. The oligonucleotide according to claim 4 wherein said oligonucleotide has the sequence:

3' TGG-TGT-GTC-GTA-CCG-TTG-CTG 5'.

6. The oligonucleotide according to claim 1 wherein said antigen is EBNA-1.

7. A method of inhibiting expression of a viral antigen comprising contacting said virus with said oligonucleotide according to claim 1, under conditions such that said inhibition is effected.

8. The method according to claim 7 wherein said virus is EBV and said oligonucleotide is contacted with messenger RNA for said antigen, which antigen is an EBV antigen involved in viral replication, said oligonucleotide having a sequence such that it hybridizes with said messenger RNA, under conditions such that said hybridization is effected and said viral replication thereby inhibited.

9. Use of the oligonucleotide of claim 1 to treat a viral infection wherein a virally infected cell is contacted with said oligonucleotide.

10. Use according to claim 9 wherein said virally infected cells are infected with an Epstein-Barr virus and said oligonucleotide is contacted with messenger RNA for an antigen, wherein said antigen is an Epstein-Barr virus antigen required for viral replication or for maintenance of the genome of the Epstein-Barr virus within said infected cell, and wherein said oligonucleotide has a sequence such that it hybridizes with said messenger RNA under conditions such that said hybridization is effected and expression or maintenance is thereby inhibited.

11. A pharmaceutical composition, in dosage unit form, comprising said oligonucleotide according to claim 1, in an amount sufficient to effect said selective inhibition, together with a pharmaceutically acceptable carrier.

12. The composition according to claim 11 wherein said oligonucleotide has the sequence

3' TGG-TGT-GTC-GTA-CCG-TTG-CTG 5'.

13. The composition according to claim 11 wherein said composition is in the form of a lotion, gel, cream, foam or ointment.

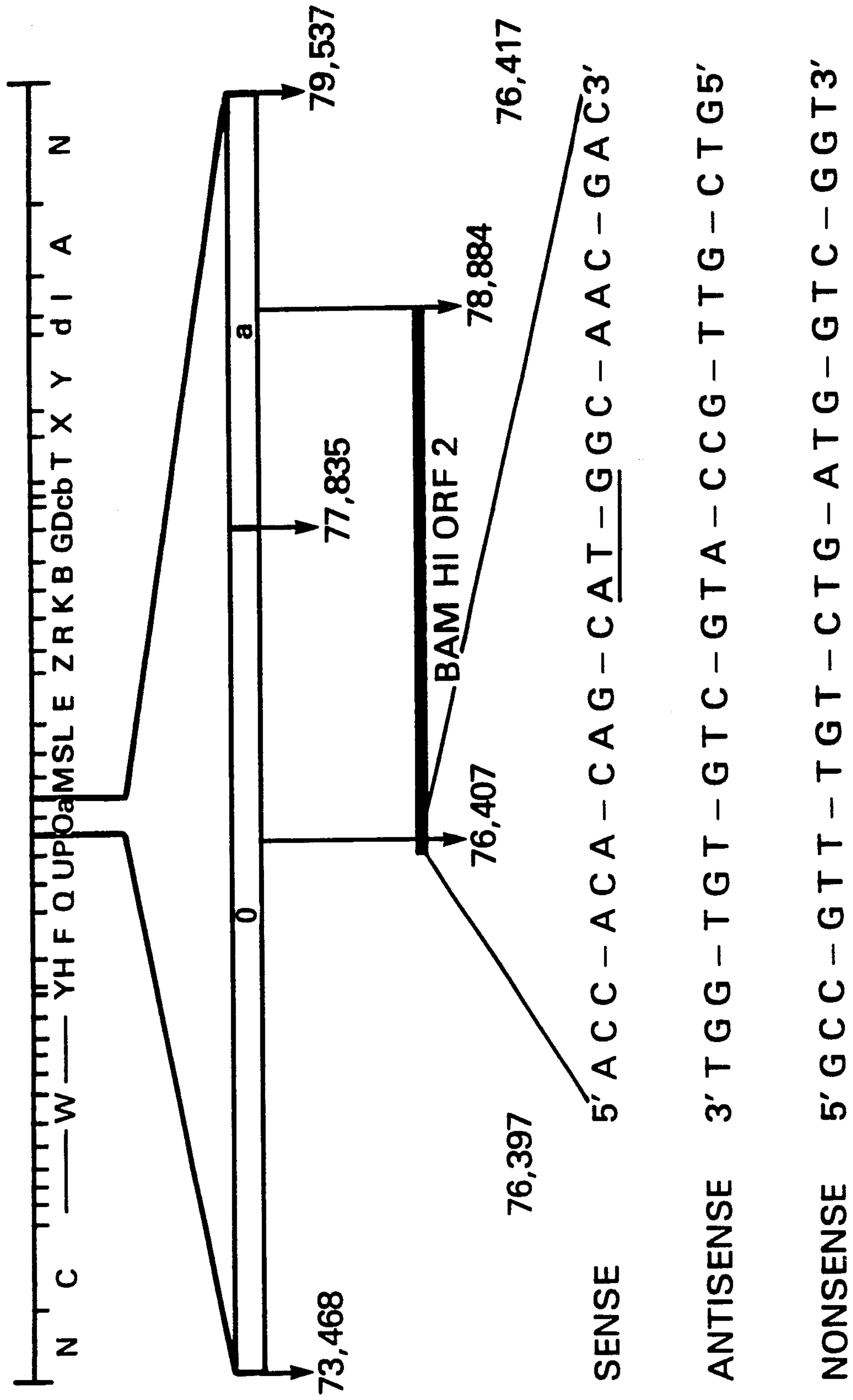
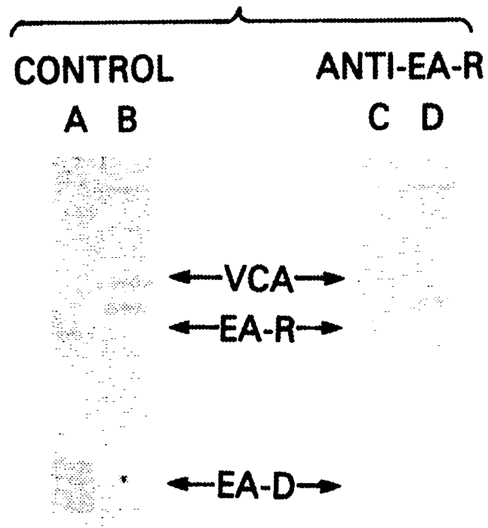


FIG. 1
N C W YH F Q UP Oa MSL E Z R K B GDcb T X Y d I A N
0
a
73,468
79,537
77,835
78,884
76,407
BAM HI ORF 2
76,397
76,417
SENSE 5' A C C – A C A – C A G – C A T – G G C – A A C – G A C 3'
ANTISENSE 3' T G G – T G T – G T C – G T A – C C G – T T G – C T G 5'
NONSENSE 5' G C C – G T T – T G T – C T G – A T G – G T C – G G T 3'

FIG. 2



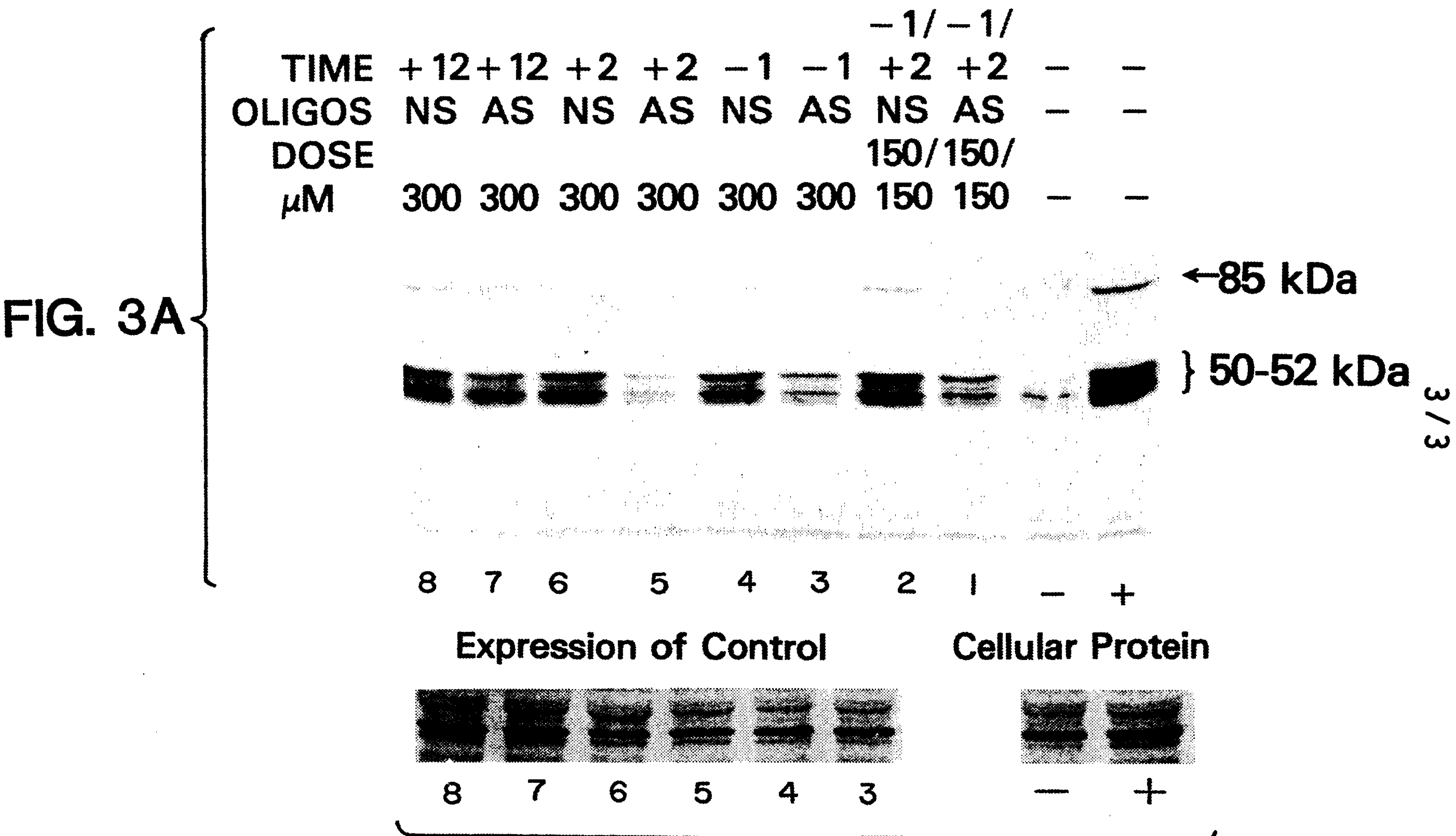


FIG. 3A

FIG. 3B

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/06734

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61K 31/70 U.S.: 514/44

II FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	514/44; 536/27; 435/172.3, 235.1

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X / Y	US,A, 4,689,320 (KAJI) 25 August 1987, see entire document.	1,2,7,9-11,13 / 3-6,8,12
X / Y P	US, A, 5,023,243 (TULLIS) 11 June 1991, see entire document.	1,2,7,9,11 / 3-6,8,10,12
Y	Proc. Natl. Acad. Sci., Vol. 82, issued December 1985, Speck et al., "Analysis of the transcript encoding the latent Epstein-Barr virus nuclear antigen I: A potentially polycistronic message generated by long-range splicing of several exons", pages 8305-8309, see entire document.	3-5,8,10,12
Y	Virology, Vol. 157, issued 1987, Goldschmidts et al., "A Restricted Component of the Epstein-Barr Virus Early Antigen Complex Is Structurally Related to Ribonucleotide Reductase", pages 220-226, see entire document.	6

* Special categories of cited documents: [10]

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"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
13 January 1992	24 JAN 1992
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Diane Moffett for Jacqueline Stone

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