



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁴ : C07K 7/06, 7/08, 7/10 C07K 15/12, A61K 39/395 G01N 33/53, C12N 15/00	A1	(11) International Publication Number: WO 87/ 06940 (43) International Publication Date: 19 November 1987 (19.11.87)
(21) International Application Number: PCT/US87/01046 (22) International Filing Date: 5 May 1987 (05.05.87) (31) Priority Application Number: 860,276 (32) Priority Date: 6 May 1986 (06.05.86) (33) Priority Country: US (71) Applicant: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 2199 Addison Street, Berkeley, CA 94720 (US). (71)(72) Applicant and Inventor: SOUZA, Lawrence, M. [US/US]; 1302 Witherspoon Drive, Thousand Oaks, CA 91360 (US). (72) Inventor: SLAMON, Dennis, J. ; 23122 Calvert Street, Woodland Hills, CA 91367 (US).	(74) Agent: HESLIN, James, M.; Townsend and Townsend, One Market Plaza, 2000 Steuart Tower, San Francisco, CA 94105 (US). (81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent). Published <i>With international search report.</i>	
(54) Title: ASSAYS AND ANTIBODIES FOR N- <i>myc</i> PROTEINS (57) Abstract Methods and compositions for identifying patients suffering from cancer, particularly neural and neuroendocrine cancers. It has been found that the protein expression product of the human N- <i>myc</i> proto-oncogene may be detected in certain biological specimens, particularly tissue specimens and sputum samples. By obtaining immunogenic N- <i>myc</i> polypeptides, either synthetically or by isolation from a natural source, antibodies specific for the N- <i>myc</i> protein are obtained. Those antibodies may then be used in immunological techniques for detecting the presence of N- <i>myc</i> in the biological samples. In particular, the antibodies may be employed in immunohistochemical techniques to detect the N- <i>myc</i> protein in prepared tissue and sputum samples.		

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ASSAYS AND ANTIBODIES FOR N-myc PROTEINSBACKGROUND OF THE INVENTION1. Field of the Invention

5 Oncogenes are genes that, when activated or altered, may be involved in transformation of normal cells to a neoplastic phenotype. Numerous oncogenes have been identified in both humans and animals, and the transformation mechanisms associated with each vary
10 widely. Frequently, the oncogene has a corresponding normal cellular gene referred to as a proto-oncogene, and modification of either the expression or the structure of the proto-oncogene may result in neoplastic transformation. For example, alteration of
15 a single nucleotide in the ras proto-oncogene renders the host cell neoplastic, while transposition of the c-myc proto-oncogene results in increased expression and neoplastic transformation of the host. Another such example would be translocation of the c-abl
20 proto-oncogene which results in a unique transcript and protein found in chronic myelogenous leukemia (CML).

N-myc is a human proto-oncogene which was originally identified because of its similarity to the viral c-myc oncogene. Gene amplification and/or
25 increased expression of N-myc has been found in certain tumor cell lines as well as both primary and metastatic tumors. In particular, N-myc has been associated with neuroblastomas, retinoblastomas, and small-cell lung cancers (SCLC). Additionally, expression of N-myc has
30 been found to complement mutant ras genes in tumorigenic conversion of rat fibroblasts. Thus, N-myc appears to be involved in the pathogenesis of certain human malignancies.

 It would be desirable to isolate and characterize the N-myc gene product and to provide assay
35 methods and reagent compositions useful for identifying and diagnosing tumors in which N-myc is expressed. In

particular, it would be desirable to obtain antigenic reagents capable of eliciting antibodies specific for the N-myc gene product and to use such antibodies in diagnosis and treatment of N-myc related cancers.

5 2. Description of Pertinent References

N-myc proto-oncogenes were first identified in human neuroblastoma cell lines which showed a 25 to 700-fold amplification of the gene. Schwab et al. (1983) Nature 305:245, and Kohl et al. (1983) Cell
10 35:359. The sequence of the N-myc gene is reported in Kohl et al. (1986) Nature 319:73. Amplification and increased transcription of N-myc in untreated primary neuroblastomas and retinoblastomas are reported in Brodeur et al. (1984) Science 224:1121; Seeger et al.
15 (1985) New Engl. J. Med. 313:1111; and Lee et al. (1984) Nature 309:458. Amplification and increased transcription of N-myc have also been observed in human small-cell lung cancers. Nau et al. (1986) Proc. Natl. Acad. Sci. USA 83:1092-1096.

20 SUMMARY OF THE INVENTION

The present invention provides methods and compositions for identifying and monitoring human cancers, particularly cancers which have neural or neuroendocrine properties such as neuroblastomas,
25 retinoblastomas, and small-cell lung cancers. The methods rely on detection of N-myc protein in a biological specimen, usually a cell sample such as a tissue sample or sputum sample. Presence of the N-myc protein in the biological specimen may be diagnostic
30 and/or prognostic of the cancer.

Polypeptides and antibodies thereto are utilized for detecting the N-myc proteins, where the polypeptides are associated with immunogenic sites on the protein. The polypeptides may be natural or
35 synthetic and will include at least six contiguous amino acids of the natural protein, usually including at least 9 contiguous amino acids, more usually

including at least 12 contiguous amino acids, representing detectable epitope(s) on the N-myc protein. Such polypeptides include the N-myc protein in substantially pure form as well as fragments thereof. Monoclonal or polyclonal antibodies against the polypeptides are prepared by conventional techniques, and the antibodies and polypeptides are utilized in a variety of conventional immunologic assay and histochemical staining techniques for detection of N-myc protein in the biological specimen.

The present invention is particularly useful for distinguishing among morphologically similar tumor types. For example, it is frequently difficult to distinguish various round cell tumors of childhood, such as neuroblastomas, neuroepitheliomas, Ewing sarcomas, rhabdomyosarcomas, and lymphomas. Since the treatment required depends greatly on the nature of the tumor, it is important to be able to distinguish the tumor type at an early stage. Use of the present invention will allow early identification of neuroblastomas and other tumors expressing N-myc. The present invention will also be useful for identifying the cellular origin of micrometastases in non-primary sites such as lymph nodes and bone marrow, and the amount of the N-myc protein expressed, measured by the intensity of staining, may correlate with the disease prognosis. The present invention also promises to provide early detection of small cell lung cancers, particularly by detection of cancer cells in sputum and/or lung lavage samples.

In a particular aspect of the present invention, novel DNA molecules are provided for expressing the N-myc polypeptides. The DNA molecules include at least a portion of the N-myc gene under the transcriptional control of a heterologous promoter, and usually also include an origin of replication. The N-myc gene may be natural or synthetic, and the DNA

molecule expressed in vitro to produce the N-myc polypeptides.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 represents the nucleotide sequence
5 together with the deduced amino acid sequence for the N-myc 1 clone described in the Experimental section hereinafter.

Fig. 2 illustrates the P414/936 plasmid used
in expressing various N-myc 1 fragments, as described
10 in detail in the Experimental section hereinafter.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

According to the present invention, methods
for identifying cancers in human patients rely on
detection of the expression product of the human N-myc
15 proto-oncogene in a biological specimen, typically body fluids, tissue, or sputum samples. Conveniently, detection is accomplished by immunological techniques, such as immunohistochemical staining of a cell sample employing either monoclonal or polyclonal antibodies
20 specific for the N-myc protein. Detection of the N-myc protein is particularly useful in distinguishing specific cancers, such as neuroblastomas, from morphologically similar cancers and for the early detection and differential diagnosis of cancers, such
25 as small-cell lung cancers.

N-myc is a human proto-oncogene which has
been associated with the pathogenesis of a number of
human tumors, particularly neural and neuroendocrine
associated tumors such as neuroblastomas,
30 retinoblastomas, and small-cell lung cancers. As demonstrated by the work reported herein, the expression product of the N-myc gene is a doublet phosphoprotein including polypeptides of about 62 kD and 64 kD, respectively, where it is believed that the
35 62 kD species represents the gene product prior to phosphorylation. These molecular weights, of course, are only approximate and subject to experimental error

in the measurement techniques reported in the Experimental section hereinafter. The protein has a half-life of about 30 to 50 minutes, and is located within the cell nucleus where 40 to 50% is associated with the nuclear matrix. As further demonstrated herein, neoplastic transformation of certain neural and neuroendocrine associated cells is characterized by detectable expression of the N-myc protein.

The polypeptides of the present invention will be either haptenic or antigenic, including at least 6 amino acids, usually at least 9 amino acids, and more usually 12 or more amino acids found contiguously within the natural N-myc protein. The contiguous amino acids may be located within any region of either of the doublet polypeptides and will correspond to at least one epitopic site which is characteristic of the protein. By characteristic, it is meant that the epitopic site will allow immunologic detection of the N-myc protein in a cell sample with reasonable assurance, in most cases allowing N-myc to be immunologically distinguished from other related proteins, such as c-myc. In other cases, however, the epitopic site(s) will be cross-reactive with other proteins, such as c-myc.

As reported in greater detail in the Experimental section hereinafter, six particular polypeptides within the carboxyl terminal 394 amino acids of the putative N-myc protein (as reported by Kohl et al. (1986), supra.) have been identified which are capable of eliciting antibodies useful in the present invention. The sequences of these polypeptides (using the standard single letter designation) are as follows:

	(I)	AFGLGGLGGL LERAVSEKLQ	TPNPFVILQDC HGRG;	MWSGFSAREK
35	(II)	ELAHPAAECEV APASAPAAGP RPGGRQTSQG	DPAVVFPFPV AVASGAGIAA DHKALS;	NKREPAPVPA PAGAPGVAPP

	(III)	ELAHPAECV APASAPAAGP RPGGRQTSGG DEEEDEEEI;	DPAVVFPPV AVASGAGIAA DHKALSTSGE	NKREPAPVPA PAGAPGVAPP DTLSDSDDED
5	(IV)	GEDTLSDSDD RSSSNTKAVT	EDDEEEDEEE TFTITVRPKN	EIDVVTVEKR AALGP;
	(V)	GRAQSSELIL SEDAPPQKKI SPRNSDSEDS FLTLRDHVP; and	KRCLPIHQH KSEASPRPLK ERRRNHNILE	NYAAPSPYVE SVIPPKAKSL RQRRNDLRSS
10	(VI)	ELVKNEKAAK LLEKEKLQA	VVILKKATEY RQQQLLKKIE	VHSLQAEHHQ HARTC.

Of these six polypeptides, it has been found that II and III are capable of eliciting antibodies having very high specificity and affinity for the N-myc protein and which are free from reactivity with c-myc and other proteins. In contrast, antibodies raised against polypeptide V appear to cross-react with c-myc protein. While antibodies raised against all six polypeptides react with N-myc in immunoprecipitation assays with lysates of neuroblastoma cell lines, only II, III, and V elicit antibodies which histochemically stain the whole cells under the conditions tested.

The N-myc polypeptides may be natural, i.e., the entire N-myc protein or fragments thereof isolated from a natural source, or may be synthetic. The natural polypeptides may be isolated from natural sources, such as neuroblastoma and retinoblastoma cell lines known to produce the N-myc protein, by conventional techniques such as affinity chromatography. Conveniently, polyclonal or monoclonal antibodies obtained according to the present invention may be used to prepare a suitable affinity column by well known techniques. Such techniques are taught, for example, in Hudson and Hay, Practical Immunology, Blackwell Scientific Publications, Oxford, United Kingdom, 1980, Chapter 8.

Synthetic polypeptides which are immunologically cross-reactive with the natural N-myc protein may be produced by either of two general approaches. First, polypeptides having fewer than about 50 amino acids, more usually fewer than about 20 amino acids, may be synthesized by the well-known Merrifield solid-phase synthesis method where amino acids are sequentially added to a growing chain (Merrifield (1963) J. Am. Chem. Soc. 85:2149-2156).

The amino acid sequences of such synthetic polypeptides may be based on the sequence of Fig. 1 described in the Experimental section hereinafter, or on the sequence for the entire N-myc gene reported in Kohl et al. (1986), supra.

The second and preferred method for synthesizing the polypeptides of the present invention involves the expression in cultured cells of recombinant DNA molecules encoding a desired portion of the N-myc gene. The N-myc gene may itself be natural or synthetic with the natural gene obtainable from cDNA or genomic libraries using available probes, such as pNb-1 (Schwab et al. (1983) Nature 305:245). Alternatively, probes may be synthesized based on the DNA sequences reported in Fig. 1, as described in the Experimental section hereinafter. Suitable cDNA and genomic libraries may be obtained from human cell lines known to contain the N-myc gene, such as the LA-N-5 and IMR-32 human neuroblastoma cell lines. Alternatively, polynucleotides may be synthesized by well-known techniques. For example, short single-stranded DNA fragments may be prepared by the phosphoramidite method described by Beaucage and Carruthers (1981) Tett. Letters 22:1859-1862. A double-stranded fragment may then be obtained either by synthesizing the complementary strand and annealing the strands together under appropriate conditions or by adding the complementary strand using DNA polymerase with an

appropriate primer sequence. Particular DNA sequences may be based on Fig. 1 herein, or on the sequence reported by Kohl et al. (1986), supra.

The natural or synthetic DNA fragments coding
5 for a desired N-myc fragment will be incorporated in DNA constructs capable of introduction to and expression in an in vitro cell culture. Usually, the DNA constructs will be suitable for replication in a unicellular host, such as yeast or bacteria, but may
10 also be intended for introduction and integration within the genome of cultured mammalian or other eukaryotic cell lines. DNA constructs prepared for introduction into bacteria or yeast will include a replication system recognized by the host, the N-myc
15 DNA fragment encoding the desired polypeptide product, transcriptional and translational initiation regulatory sequences joined to the 5'-end of the N-myc DNA sequence, and transcriptional and translational termination regulatory sequences joined to the 3'-end of the
20 N-myc sequence. The transcriptional regulatory sequences will include a heterologous promoter which is recognized by the host. Conveniently, available expression vectors which include the replication system and transcriptional and translational regulatory
25 sequences together with an insertion site for the N-myc DNA sequence may be employed.

To be useful in the detection methods of the present invention, the polypeptides are obtained in substantially pure form, that is, typically about 50%
30 w/w or more purity, substantially free of interfering proteins and contaminants. Preferably, the N-myc polypeptides are isolated or synthesized in a purity of at least about 80% w/w and, more preferably, in at least about 95% w/w purity. Using conventional protein
35 purification techniques, homogeneous polypeptides of at least 99% w/w can be obtained. For example, the proteins may be purified by use of the antibodies

described hereinafter using immunoadsorbent affinity chromatography. Such affinity chromatography is performed by first linking the antibodies to the solid support and then contacting the linked antibodies with the source of the N-myc proteins, e.g., lysates of cells which naturally produce N-myc or which produce N-myc as a result of introduction of a recombinant N-myc DNA molecule.

Once a sufficient quantity of N-myc polypeptide has been obtained, polyclonal antibodies specific for the N-myc protein may be produced by in vitro or in vivo techniques. In vitro techniques involve in vitro exposure of lymphocytes to the antigenic polypeptides, while in vivo techniques require the injection of the polypeptides into a wide variety of vertebrates. Suitable vertebrates are non-human, including mice, rats, rabbits, sheep, goats, and the like. Polypeptides having more than about 30 amino acids, particularly more than about 50 amino acids, may serve directly as immunogens. If the polypeptide is smaller than about 10 kD, particularly less than about 6 kD, it may be necessary to join the polypeptide to a larger molecule to elicit the desired immune response. The immunogens are then injected into the animal according to the predetermined schedule, and the animals are bled periodically with successive bleeds having improved titer and specificity. The injections may be made intramuscularly, intraperitoneally, subcutaneously, or the like, and usually an adjuvant, such as incomplete Freund's adjuvant, will be employed.

If desired, monoclonal antibodies can be obtained by preparing immortalized cell lines capable of producing antibodies having the desired specificity. Such immortalized cell lines may be produced in a variety of ways. Conveniently, a small vertebrate, such as a mouse, is hyperimmunized with the desired

antigen by the method just described. The vertebrate is then killed, usually several days after the final immunization, the spleens removed, and the spleen cells immortalized. The manner of immortalization is not
5 critical. Presently, the most common technique is fusion with a myeloma cell fusion partner, as first described by Kohler and Milstein (1976) Eur. J. Immunol. 6:511-519. Other techniques include EBV transformation, transformation with bare DNA, e.g.,
10 oncogenes, retroviruses, etc., or any other method which provides for stable maintenance of the cell line and production of monoclonal antibodies.

When employing fusion with a fusion partner, the manner of fusion is not critical and various
15 techniques may be employed. Conveniently, the spleen cells and myeloma cells are combined in the presence of a nonionic detergent, usually polyethylene glycol, and other additives such as Dulbecco's Modified Eagle's Medium, for a few minutes. At the end of the fusion,
20 the nonionic detergent is rapidly removed by washing the cells. The fused cells are promptly dispensed in small culture wells (usually in a microtiter plate) at relatively low density, ranging from about $1-5 \times 10^5$ per well, in a selective medium chosen to support growth of
25 the hybrid cells while being lethal to the myeloma cells. Usually, the myeloma cell line has been mutated to be sensitive to a lethal agent, typically being HAT sensitive.

After sufficient time, usually from one to
30 two weeks, colonies of hybrids are observed and plates containing hybrid positive wells are identified. The plates and wells having only one colony per well are selected, and supernatants from these wells are tested for binding activity against the N-myc protein or the
35 isolated antigen. Once positive hybridomas are identified, the cell line can be maintained as viable cultures and/or by lyophilization and frozen storage.

Depending on the desired use for the antibodies, further screening of the hybridomas may be desirable. Hybridomas providing high titers are desirable. Furthermore, cytotoxic antibodies, e.g.,
5 IgG2a, IgG2b, IgG3 and IgM, may be selected for use in therapeutic treatment of colorectal cancers. For use in immunodiagnostic assays, antibodies having very high specificity for the antigenic site are desirable.

Once the desired hybridomas have been
10 selected, monoclonal antibodies may be isolated from the supernatants of the growing colonies. The yield of antibodies obtained, however, is usually low. The yield may be enhanced by various techniques, such as injection of the hybridoma cell line into the
15 peritoneal cavity of a vertebrate host which accept the cells. Monoclonal antibodies may then be harvested from the ascites fluid or the blood. Proteinaceous and other contaminants will usually be removed from the monoclonal antibodies prior to use by conventional
20 technique, e.g., chromatography, gel filtration, precipitation, extraction, or the like.

By properly selecting polypeptides used as the immunogen, antibodies having high specificity and affinity for the N-myc protein can be obtained. The
25 polypeptide selected should represent one or more epitopic sites which are unique to the N-myc protein and which can distinguish N-myc from closely related proteins such as c-myc. Such unique epitopes are found on polypeptides II and III shown above.

30 The polypeptides and antibodies of the present invention may be used with or without modification. Frequently, the polypeptides and antibodies will be labelled by joining, either covalently or non-covalently, a substance which provides for a
35 detectable signal. A wide variety of labels and conjugation techniques are known and are reported extensively in both the scientific and patent

literature. Suitable labels include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescers, chemiluminescers, magnetic particles, and the like. Patents teaching the use of such labels include U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241.

Antibodies and polypeptides prepared as described above can be used in various immunological techniques for detecting N-myc protein in biological specimens, particularly cell samples such as neurocytes, retina cells, and small lung cells (neuroendocrine-derived) and body fluid samples, including blood, plasma, serum, urine, and the like. Depending on the nature of the sample, both liquid phase assays and solid-phase immunohistochemical staining techniques will find use. Conveniently, immunohistochemical staining techniques may be used with cell samples including tissue samples, sputum, and lung lavage samples. For example, a tissue sample may be fixed in formalin, B-5, or other standard histological preservative, dehydrated and embedded in paraffin as is routine in any hospital pathology laboratory. Sections may then be cut from the paraffinized tissue block and mounted on glass slides. The N-myc protein, if present, may then be detected in the nucleus by exposure with labelled anti-N-myc antibody or exposure to unlabelled anti-N-myc antibody and a labelled secondary antibody. Sputum and lavage samples are typically prepared in a similar manner where the sample is first dehydrated by exposure to a dehydrating agent, typically a low molecular weight alcohol.

Liquid phase immunoassays or Western Blot analysis will also find use in the detection of the N-myc protein particularly in body fluids when the protein is shed into such fluids, e.g., blood or urine.

Solid tissue and sputum samples may also be assayed in liquid phase systems by lysing the cellular sample in order to release the intracellular protein. Once the protein is released, the sample will be placed in a suitable buffer, the sample buffer subjected to a suitable immunoassay. Numerous competitive and non-competitive immunoassays are available and described in the scientific and patent literature.

The antibodies of the present invention may also find use in cancer therapy and other medical applications. For example, anti-N-myc antibodies, preferably human antibodies, may be coupled to toxins, such as diphtheria toxin and the ricin A chain, and administered to patients suffering from neural and neuroendocrine cancers. The use of antibody conjugated toxins in cancer therapy is described generally in U.S. Patent Nos. 4,093,607; 4,340,535; 4,379,145; and 4,450,154. Antibodies alone may also find use in treatment, particularly by blocking or interrupting the activity of the N-myc protein which contributes to the neoplastic phenotype.

The following experiments are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

1. Preparation and Expression of Partial cDNA Clone of N-myc.

To characterize the N-myc gene and its gene product, a cDNA library was constructed from the LA-N-5 human neuroblastoma cell line (insert citation) using the Okayama-Berg vector (Okayama and Berg (1982) Mol. Cell Biol. 2:101). Approximately 80,000 clones were screened with pNb-1 which is a plasmid containing a human N-myc fragment isolated from a neuroblastoma cell line (Schwab et al. (1983), Supra.). One positive clone, designated N-myc 1, was obtained. Dideoxy sequencing of N-myc 1 was performed on independent M13 clones containing either overlapping fragments or

complementary DNA strands. The nucleotide sequence together with the deduced amino acid sequence is shown in Fig. 1. The amino acids in Fig. 1 are numbered corresponding to the complete sequencing as reported by
 5 Kohl et al. (1986) Supra.

The largest open reading frame in N-myc 1 is 1182 nucleotides long and would encode the carboxyl terminal 394 amino acids of the putative N-myc protein. Comparison of the N-myc 1 sequence and that reported by
 10 Kohl et al. (1986) Supra. reveals only one notable difference between the large open reading frame from the N-myc 1 clone and the published sequence. The third base of the codon for amino acid 226 in the N-myc 1 clone is a cytosine, while the first base of the next
 15 codon is a guanine. The published sequence shows a reversal of these two residues. This difference results in a change in amino acid 227; i.e., alanine instead of proline.

A series of vectors for expressing portions
 20 of the putative N-myc encoded protein in E. coli were constructed by fusing the 5'-region of a bovine growth hormone gene (bGH) in frame, to six different regions of the N-myc 1 clone as follows:

25	<u>Fusion Gene</u>	<u>Amino Acid Nos.*</u>	<u>Symbol*</u>
	bGH/N-myc I	96-111	_____
	bGH/N-myc II	178-253	-----
	bGH/N-myc III	178-277	
	bGH/N-myc IV	226-280	-----
30	bGH/N-myc V	311-409	~~~~~
	bGH/N-myc VI	410-464	-----

See Fig. 1.

The three sites in the bGH gene used for the fusion to N-myc specific sequences were SstI, PstI, and HindIII, generating fusion proteins containing 76, 92, and 114 residues of the amino terminus of bGH, respectively.

5 Expression vector p414/936 (insert citation), a modified temperature-sensitive runaway plasmid containing transcription termination and translation termination signals in all three reading frames adjacent to a polylinker used for introduction of genes
10 linked to a tryptophan synthetase promoter (Fig. 2), was used for bGH/N-myc constructions I, III, IV, V and VI. The basic components of the p414/936 vector are: 1) a selectable drug marker, Amp^R; 2) transcription termination signal, and 3) a poly linker containing
15 translation termination signals in all three reading frames shown in boxes. All five fusion genes expressed using this vector were promoted by a tryptophan synthetase promoter depicted by the black box. The bGH/N-myc genes, including the promoter, were all
20 cloned into the EcoRI site 5' to the promoter and at either the HpaI, BamHI or SstII sites (underlined) at the 3'-end of the genes. The specific 3'-cloning site is shown in brackets beside each construct. The shaded portion of each bar construct represents the bGH
25 domain, while the open portion represents the N-myc domain. The number of amino acids representing either bGH or N-myc in each construct is shown in brackets within each bar construct. In addition, the letters opposite the bracketed numbers in the shaded portion
30 refer to the restriction endonuclease sites in bGH where the N-myc gene segments were fused: H (HindIII), S (SstI), and P (PstI). bGH/N-myc I was constructed by cloning the MluI/BamHI fragment (Fig. 1) of N-myc into M13mp20. The N-myc fragment was then recovered by
35 cleavage with HindIII and SstII for cloning into the p414/936 expression vector with the appropriate bGH fragment (EcoRI/HindIII). bGH/N-myc III was generated

by cleaving N-myc 1 with TagI and blunt ending the site by filling in the appropriate nucleotides with Klenow. N-myc 1 was then cut with SstI and the N-myc fragment ligated to the vector as shown above. bGH/N-myc IV was constructed by cloning the small N-myc MspI fragment into M13mp10 (AccI) and recovering it by cleavage with PstI and BamHI. bGH/N-myc V was formed in the same manner as bGH/N-myc IV, using the largest MspI fragment of N-myc 1. Finally, bGH/N-myc VI was generated using an MspI/HindIII fragment of N-myc 1 cloned into M13mp8 (AccI/HindIII). The fragment was recovered using HindIII and HindII for cloning into the vector. The dotted portion of the bar construction for bGH/N-myc VI represents the 3'-untranslated sequence present in this construction.

Fusion gene bGH/N-myc II was expressed using vector pCFM414 previously described for obtaining expression of the human c-myc antigen. bGH/N-myc II was constructed by ligating an SstI/RsaI fragment of N-myc 1 in frame with the SstI site in the bGH gene and allowing the encoded product to terminate at a stop codon provided in the vector.

The above-described vectors were then expressed in E. coli as follows.

2. Preparation of Antisera in Rabbits

The bGH/N-myc fusion proteins obtained as described above were used to produce polyclonal antisera in rabbits by the method described by Slamon et al. (1985) Science 228:1427.

3. Characterization of the N-myc Protein Using the Polyclonal Antisera.

Antisera generated to the various N-myc protein fragments were tested in a liquid phase immunoprecipitation assay using human neuroblastoma cell lines (LA-N-5 and IMR-32) known to express N-myc transcripts, but not c-myc. The human promyelocytic cell line, HL-60, was used as a negative control, since

these cells express high levels of c-myc transcripts, but not N-myc. In addition, two other human cell lines, the HT 29 (colon carcinoma) and U251 (glioma), were used as negative controls since neither express

5 N-myc transcripts. The criteria used to identify a protein as N-myc encoded were two-fold. First, the protein had to be immunoprecipitated from neuroblastoma cell lysates by antisera to at least two different

10 N-myc-encoded fragments representing separate areas of the putative amino acid sequence, thus greatly reducing the possibility that the immunoprecipitation was due to chance sequence homology between the vector-expressed fragment and a cellular protein other than N-myc.

15 Second, the protein should not be found in other human tumor cell lines (including the neural derived glioma cells) where the N-myc transcript is not found.

Using these criteria, a protein appearing as a doublet of 62 kD and 64 kD was consistently immunoprecipitated from LA-N-5 human neuroblastoma

20 cells by antibody directed to each of the six N-myc fragments generated in E. coli. Similar results were seen for the IMR-32 human neuroblastoma cell line. Immunoprecipitation of this protein could be completely removed in all cases by competition with the appropriate

25 N-myc fragment, indicating a specific antigen-antibody reaction. Moreover, immunoprecipitation of the 62-64 kD doublet could not be blocked when non-homologous antibody and antigen combinations were used; i.e., attempts to block precipitation mediated by

30 antisera to fragment II with fragment I antigen were unsuccessful, again indicating that the precipitation is due to a specific antigen-antibody reaction. Finally, to ensure that the precipitation of the 62-64

35 kD protein was due to antibodies directed to the N-myc and not the bGH portion of the fusion protein, bGH alone was added to the reaction mix. In no instance

did the bGH inhibit the precipitation of the 62-64 kD protein.

The c-myc protein is similar in size (64-67 kD) and appearance (doublet) to the N-myc protein (Hann and Eisenman (1984) Mol. Cell Biol. 4:2486). Evidence that this is not the c-myc protein, however, is provided by two sets of data. First, the protein identified using the anti-bGH/N-myc fusion antibodies is found in cells (LA-N-5) which lack detectable c-myc transcripts. Second, five of the six antisera fail to identify any specific protein when used in immunoprecipitation assay with lysate from the HL-60 cell line which is rich in c-myc transcripts, indicating that they do not recognize the c-myc protein. The antisera directed against N-myc fragment V did recognize a 64-67 kD doublet in lysate from HL-60 cells on long exposure (96 hours) of the immunoprecipitation radioautograph. Comparison of the deduced amino acid sequence from this fragment of N-myc and the analogous area in the c-myc protein shows significant sequence homology which would account for the ability of this antiserum to recognize the c-myc protein. None of the anti-bGH/N-myc antisera precipitated a 62-64 kD protein in the U251 or HT-29 control cell lines. Thus, the data demonstrate that the 62-64 kD protein is the N-myc-encoded protein.

Several aspects of the biochemistry and biology of the N-myc protein were investigated using the N-myc-specific antisera. To determine if the N-myc protein was phosphorylated, LA-N-5 cells were metabolically labelled in phosphate-free media with ³²P-orthophosphate (ICN Biochemicals, Inc., Irvine, CA) by the method of Osterman, in: Methods of Protein and Nucleic Acids Research, Springer-Verlag, New York, New York, 1984, pp. 144-150. The lysate was then subjected to immunoprecipitation using antisera to the bGH/N-myc II fusion protein and analyzed on SDS-PAGE gel. A band at 62-64 kD was found to be labelled with

³²P-orthophosphate. Thus, like c-myc, the N-myc protein is a phosphoprotein.

The level of N-myc protein synthesis in LA-N-5 neuroblastoma cells compared to other cellular proteins was estimated using an immunoprecipitation assay. In these experiments, a known amount of radioisotope-labelled whole cell lysate was subjected to immunoprecipitation using a combination of N-myc-specific antisera (anti-bGH/N-myc II and IV). The resulting precipitated proteins were then subjected to SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel) analysis, and the doublet band at 62-64 kD was eluted from the gel, counted and compared to the total trichloroacetic acid-precipitable counts in the whole cell lysate as described by Slamon et al. (1985), *supra*. Approximately 0.076% of the total ³⁵S-methionine incorporation in these cells was associated with the 62-64 kD N-myc protein.

The kinetics of intracellular turnover of the N-myc protein were determined by pulse-chase labelling experiments. LA-N-5 human neuroblastoma cells were labelled with ³⁵S-methionine for sixty minutes and chased with excess unlabelled methionine (Slamon et al. (1985), *supra*.). The half-life of the p62-64^{N-myc} doublet was between 30-50 minutes. Again, this feature of the N-myc protein is shared with c-myc, which has a half-life of 20-30 minutes.

To determine the subcellular localization of the N-myc protein, each of anti-N-myc antisera were used in immunocytochemical analyses of LA-N-5 cells. Using an indirect immunoperoxidase staining procedure and dilutions of N-myc antisera of 1:2000 to 1:4000 by volume, a strong staining reaction was noted in the nuclei of the cells. All cells showed nuclear staining; however, variable intensity of the stain from cell to cell, indicating heterogeneity in N-myc protein content. While all six of the anti-N-myc antisera

recognized the 62-64 kD protein in LA-N-5 cells by immunoprecipitation assay, under the conditions tested only three of these sera stained the neuroblastoma cells histochemically, anti-N-myc II, III and V. The fact that anti-N-myc sera I, IV and VI did not react immunocytochemically could be due to fixative-mediated alterations of the antigenic determinants to which the non-reacting antisera are directed. Conversely, these regions of the protein may be unavailable for recognition by the particular antisera, due to interactions of the antigenic regions with other cellular macromolecules or to the tertiary structure of N-myc within intact cells. To determine the specificity of the cytochemical reaction, HL-60 cells were used as a control cell line. Two of the three antisera (bGH N-myc II and III) did not stain HL-60 cells, indicating specificity for the N-myc protein. The antisera directed against N-myc fragment V stained both LA-N-5 and HL-60 cells. As with the immunoprecipitation data, the ability of this antiserum to immunocytochemically recognize both the N-myc and c-myc proteins is likely due to the high degree of homology between the two proteins in this region.

The nuclear localization of the 62-64 kD protein was confirmed by biochemical fractionation of LA-N-5 cells into nuclear, cytoplasmic and membrane fractions as described by Slamon et al. (1985) supra. The data placed the majority of the N-myc protein within the nucleus of neuroblastoma cells, and thus were consistent with those obtained by immunocytochemical analysis. Experiments to evaluate the sub-nuclear localization of the N-myc protein were done by obtaining intact nuclei from LA-N-5 cells and subjecting them to differential fractionation. The nuclei were fractionated into three components; nucleoplasm, chromatin and nuclear matrix as described by Boyle et al. (1985) Mol. Cell Biol. 5:3017. These fractions

were then separately tested in immunoprecipitation assays using the N-myc specific anti-N-myc II antiserum. The majority of the N-myc protein was found associated with the nuclear matrix. Again, this is a feature which N-myc shares in common with c-myc.

Finally, to demonstrate the clinical diagnostic potential of the N-myc antisera, sections of primary human neuroblastoma tumors were tested using immunohistochemical techniques. In these experiments, only N-myc-specific antisera were used; i.e., anti-N-myc fragment II. As with cultured cell staining, the antiserum was used at dilutions of 1:2000 to 1:4000. Tissue from two separate primary tumors were examined; one from a patient with stage II disease, and one with stage IV disease. In both instances, there was intense and specific staining of malignant neuroblasts, with virtually no staining of the adjacent vascular or stromal tissue. The tumor from the patient with stage II disease was stroma rich, with nodular areas of undifferentiated cells. This tumor was shown to contain one copy of the N-myc oncogene by Southern Blot analysis (Seeger et al. (1985), Supra). The tumor from the patient with stage IV disease consisted of predominantly undifferentiated neuroblasts with surrounding vascular and stromal elements. It was shown to contain 200 copies of the N-myc oncogene (Id.)

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A polypeptide produced by expression in cultured cells of at least a portion of the human N-myc gene.

2. A polypeptide as in claim 1, wherein the portion of the human N-myc gene corresponds to amino acids at the carboxyl end of the N-myc protein.

3. Antigenic or haptenic polypeptides comprising at least six amino acids but fewer than all amino acids of the N-myc protein.

4. Polypeptides as in claim 3, having an amino acid sequence selected from the group consisting of:

(I)	AFGLGGLGGL LERAVSEKLQ	TPNPVILQDC HGRG;	MWSGFSAREK
(II)	ELAHPAAE CV APASAPAAGP RPGGRQTS GG	DPAVVFPFPV AVASGAGIAA DHKALS;	NKREPAPVPA PAGAPGVAPP
(III)	ELAHPAAE CV APASAPAAGP RPGGRQTS GG DEEEDEEE EI;	DPAVVFPFPV AVASGAGIAA DHKALSTS GE	NKREPAPVPA PAGAPGVAPP DTLSDSDDED
(IV)	GEDTLSDSDD RSSSNTKAVT	EDDEEEDEEE TFTITVRPKN	EIDVVTVEKR AALGP;
(V)	GRAQSSELIL SEDAPPQKKI SPRNSDSEDS FLTLRDHVP; and	KRCLPIHQOH KSEASPRPLK ERRRNHNILE	NYAAPS PYVE SVIPPKAKSL RQRRNDLRSS
(VI)	ELVKNEKAAK LLEKEKLQA	VVILKKATEY RQQQLLKKIE	VHSLQAE EHQ HARTC.

5. Antibodies raised against the polypeptides of claim 3.

6. Antibodies specific for human N-myc protein.

7. A polypeptide representing an epitopic site in the human N-myc protein and having at least six contiguous amino acids found in the following sequence:

5 ELAHPAAECV DPAVVFPFPV NKREPAPVPA
 APASAPAAGP AVASGAGIAA PAGAPGVAPP
 RPPGGRQTSGG DHKALSTSGE DTLSDSDDED
 DEEEDEEEEI.

8. A method for diagnosing neoplastic
10 transformation comprising detecting human N-myc protein in a biological specimen.

9. A method as in claim 8, wherein the
15 N-myc protein is detected by exposing the cells to anti-N-myc antibodies.

10. A method for identifying neuroblastoma cells, retinoblastoma cells, and small-cell lung cancer cells, said method comprising immunologically detecting
20 the presence of human N-myc protein in a cell sample.

11. A method as in claim 10, wherein the cell sample is selected from the group consisting of neurocytes, retina cells, and small lung cells
25 (neuroendocrine-derived).

12. A recombinant DNA molecule including a structural gene coding for a polypeptide displaying N-myc antigenicity under transcriptional control of a
30 heterologous promoter.

13. A recombinant DNA molecule as in claim 12, further including an origin of replication recognized by a prokaryotic host.

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14. Substantially pure N-myc protein and fragments thereof.

15. Protein as in claim 14, having a molecular weight in the range from about 60 to 65 kD.

16. Phosphorylated protein as in claim 14.

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17. A method for treating neoplastic diseases, said method comprising administering to a patient antibodies of fragments thereof reactive with at least one epitope of the human N-myc protein.

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18. A method as in claim 17, wherein the antibodies are conjugated to a toxin.

19. A method as in claim 17, wherein the antibodies are unconjugated.

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[illegible]

2/3

thr val glu lys arg arg ser ser thr lys ala val thr thr phe thr ile thr val arg pro lys asn ala ala leu gly pro
 ACT GTG GAG AAG CCG CGT TCC TCC TCC AAC ACC ACC GCT GTC ACC ACA TTC ACC ATC ACT GTG CGT CCC AAG AAC GCA GCC CTG GGT CCC
 290 300 310

 gly arg ala gln ser ser glu leu ile leu lys arg cys leu pro ile his gln gln his asn tyr ala ala pro ser pro tyr val glu
 GGG AGG GCT CAG TCC AGC GAG CTG ATC CTC AAA CGA TGC CTT CCC ATC CAC CAG CAG CAC CAC TAT GCC GCC CCC TCC CCC TAC GTG GAG
 320 330 340

 ser glu asp ala pro pro gln lys lys ile lys ser glu ala ser pro arg pro leu lys ser val ile pro pro lys ala lys ser leu
 AGT GAG GAT GCA CCC CCA CAG AAG AAG ATA AAG AGC GAG GCG TCC CCA CGT CCG CTC AAG AGT GTC ATC CCC CCA AAG GCT AAG AGC TTG
 350 360 370

 ser pro arg asn ser asp ser glu asp ser glu arg arg arg asn his asn ile leu glu arg gln arg arg asn asp leu arg ser ser
 AGC CCC CGA AAC TCT GAC TCG GAG GAC AGT GAG GGT CGC AGA AAG CAC AAG CAC AAT CTC CTG CAG CGC CAG CGC AAC GAC CTT CGG TCG AGC
 380 390 400

 phe leu thr leu arg asp his val pro glu leu val lys asn glu lys ala ala lys val ile leu lys lys ala thr glu tyr val
 TTT CTC ACG CTC ACG GAC CAC CAC GTG CCG GAG TTG GTA AAG AAT GAG AAG CAC GCG AAC GCG AAC AAG AAA AAG GCC ACT GAG TAT GTC
 410 420 430

 his ser leu gln ala glu glu his gln leu leu glu lys glu lys leu gln ala arg gln gln gln leu leu lys lys ile glu his
 CAC TCC CTC CAG CCC GAG GAG CAC CAG CTT TTG CTG GAA AAG GAA AAA TTG CAG GCA AGA CAG CAG CAG CAG TTG CTA AAG AAA ATT GAA CAG
 440 450 460

 ala arg thr cys AM
 GCT CCG ACT TGC TAG ACGCTTCTCAAAACTGGACAGTCAGTCCACGTTTGGACATTTTGATTTTTTTTAAACAACACATTGTGTGACATTAAAGATGTTGTTACTTCAAA
 HincII

SUBSTITUTE SHEET

FIG. 1. (PART 2 OF 2)

3/3

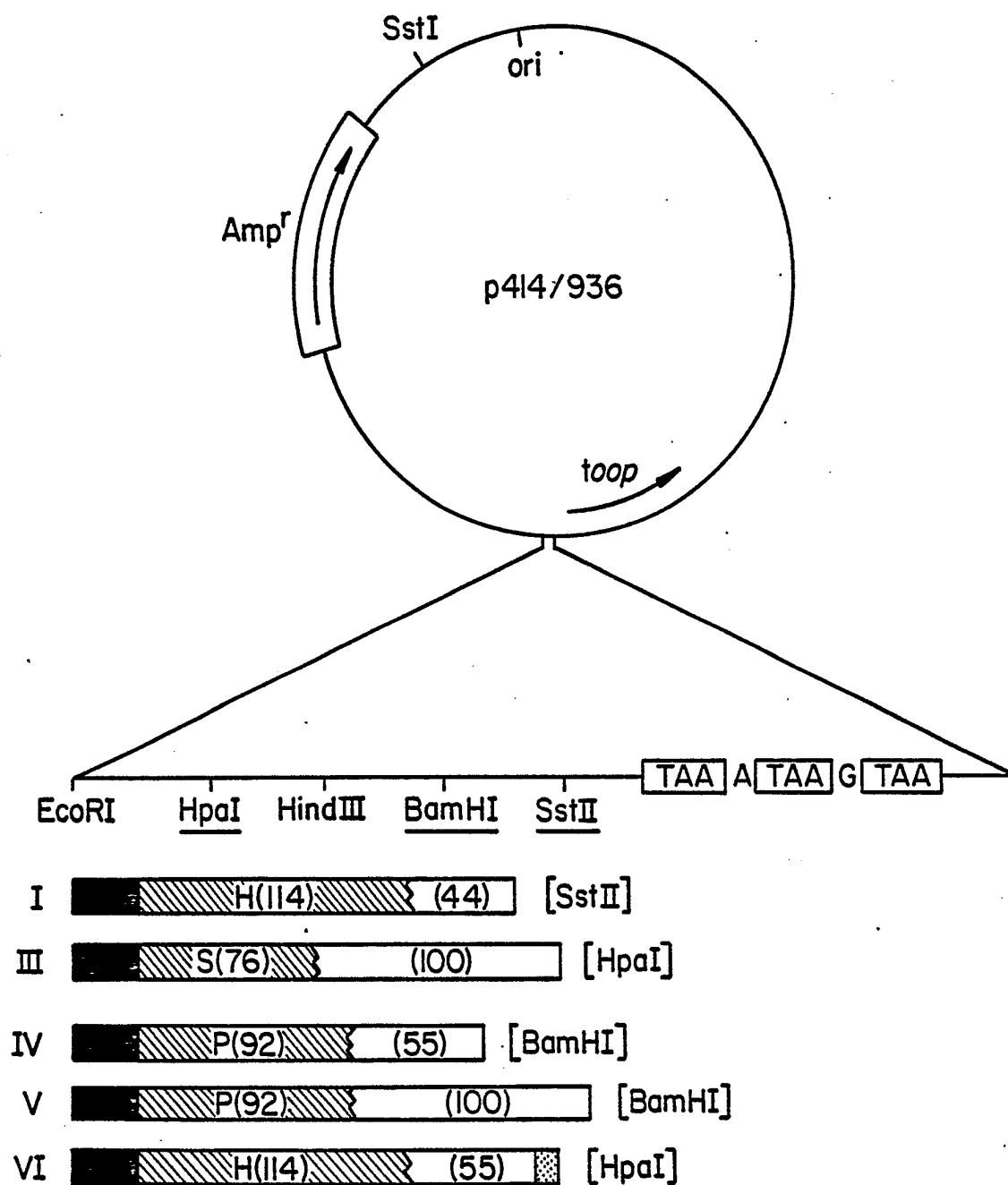


FIG. 2.

INTERNATIONAL SEARCH REPORT

International Application No. **PCT/US87/01046**

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ³		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC(4)C07K 7/06, 7/08, 7/10, 15/12; A61K39/395; G01N 33/53; C12N 15/00. U.S. 530/324, 326, 327, 328, 329, 350, 387, 402, 410, 808; 424/85; 435/7, 172.3.		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁴		
Classification System	Classification Symbols	
U.S.	530/324, 326, 327, 328, 329, 350, 387, 402, 410, 808; 424/85; 435/7, 172.3.	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁵		
CHEMICAL ABSTRACT AND BIOLOGICAL ABSTRACT ON LINE COMPUTER SEARCH		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴		
Category ⁶	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
X	PROC., NATL. ACAD. SCI. USA. VOLUME 83 ISSUED MARCH 1986, (STANTON ET AL), "NUCLEOTIDE SEQUENCE OF THE HUMAN N-MYC GENE", PAGES 1772-1776, SEE PAGE 1773, 1774, 1775 IN PARTICULAR.	1-3, 7, 12-14, 16
X, P	SCIENCE (WASHINGTON, D. C. USA), VOLUME 232 ISSUED 09 MAY 1986, (SLAMON ET AL.), "IDENTIFICATION AND CHARACTERIZATION OF THE PROTEIN ENCODED BY THE HUMAN N-MYC ONCOGENE", PAGES 768-772. SEE PAGES 768, 769, 770, 771, 772.	1-19
X	CELL, VOLUME 35, ISSUED 1983, (KOHL ET AL), "TRANSPOSITION AND AMPLIFICATION OF ONCOGENE-RELATED SEQUENCES IN HUMAN NEUROBLASTOMAS", PAGES 359-367. SEE PAGE 359 IN PARTICULAR.	10-11
X	NATURE, (LONDON, ENGLAND), VOLUME 309, ISSUED MAY 1984, (LEE ET AL), "EXPRESSION AND AMPLIFICATION OF THE N-MYC GENE IN PRIMARY RETINOBLASTOMA", PAGES 458-460. SEE PAGE 458 IN PARTICULAR.	10-11
* Special categories of cited documents: ¹⁵ "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "Z" document member of the same patent family		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search ¹		Date of Mailing of this International Search Report ²
10 JULY 1987		04 AUG 1987
International Searching Authority ¹		Signature of Authorized Officer ¹⁹
ISA/US		Christina Chan CHRISTINA CHAN

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No ¹⁸
X	NATURE, (LONDON, ENGLAND), VOLUME 305, ISSUED SEPTEMBER 1983, (SCHWAB ET AL), "AMPLIFIED DNA WITH LIMITED HOMOLOGY TO MYC CELLULAR ONCOGENE IS SHARED BY HUMAN NEUROBLASTOMA CELL LINES AND A NEURO-BLASTOMA TUMOUR, PAGES 245-248. SEE PAGES 245-246 IN PARTICULAR.	10-11
A	NATURE, (LONDON, ENGLAND), VOLUME 319, ISSUED JANUARY 1986, (KOHL ET AL), "HUMAN N-MYC IS CLOSELY RELATED IN ORGANIZATION AND NUCLEOTIDE SEQUENCE TO C-MYC", PAGES 73-77.	1-19
X	SCIENCE, (WASHINGTON, D.C., USA), VOLUME 224, ISSUED 1984, (MARX), "THE N-MYC ONCOGENE IN NEURAL TUMORS", PAGE 1088	10-11
X	PROC., NATL. ACAD. SCI. USA, VOLUME 83, ISSUED 1986, (NAU ET AL), "HUMAN SMALL-CELL LUNG CANCERS SHOW AMPLIFICATION AND EXPRESSION OF THE N-MYC GENE", PAGES 1092-1096. SEE PAGE 1092 IN PARTICULAR.	10-11
X	NEW ENGLAND JOURNAL OF MEDICINE, VOLUME 313, ISSUED OCTOBER 1985, (SEEGER ET AL), "ASSOCIATION OF MULTIPLE COPIES OF THE N-MYC ONCOGENE WITH RAPID PROGRESSION OF NEURO-BLASTOMAS", PAGES 1111-1116. SEE PAGE 1111 IN PARTICULAR.	10-11
X	SCIENCE, (WASHINGTON, D.C., USA), VOLUME 224, ISSUED 1984, (BRODEUR ET AL), "AMPLIFICATION OF N-MYC IN UNTREATED HUMAN NEURO-BLASTOMAS CORRELATES WITH ADVANCED DISEASE STAGE", PAGES 1121-1124. SEE PAGE 1121 IN PARTICULAR.	10-11
X,P	THE EMBO JOURNAL, (OXFORD, ENGLAND), VOLUME 5, ISSUED JUNE 1986, (TAYA ET AL), "NUCLEOTIDE SEQUENCE OF THE CODING REGION OF THE MOUSE N-MYC GENE", PAGES 1215-1219, SEE FIG. 3 IN PARTICULAR.	1-3, 7 12-14,16

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹⁰

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers , because they relate to subject matter ¹² not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹³, specifically:

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ¹¹

This International Searching Authority found multiple inventions in this international application as follows:

I. Claims 1-4, 7 and 14-16 drawn to a polypeptide.

II. Claims 5-6 and 17-19 drawn to an antibody and a method for treating disease.

III. Claims 8-11 drawn to a method for diagnosing neoplastic transformation. IV. Claims 12-13 drawn to a recombinant DNA.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application. Telephone practice

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☒ No protest accompanied the payment of additional search fees.