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REVIEW

Proteins Involved in Binding and Cellular Uptake of Nucleic Acids

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Abstract—The study of mechanisms of nucleic acid transport across the cell membrane is valuable both for understanding the biological function of extracellular nucleic acids and the practical use of nucleic acids in gene therapy. It has been clearly demonstrated that cell surface proteins are necessary for transport of nucleic acids into cells. A large amount of data has now been accumulated about the proteins that participate in nucleic acid transport. The methods for revealing and identification of these proteins, possible mechanisms of protein-mediated transport of nucleic acids, and cellular functions of these proteins are described.

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The regulation of gene expression influenced by intracellular nucleic acids is intensively studied in recent years [1, 2]. In particular, antisense oligonucleotides and their chemical analogs [3-5] are suggested to be useful tool for this purpose. Experiments with tissue cultures have demonstrated that antisense oligonucleotides inhibit gene expression due to complementary interactions [6-10], thus interfering with the process of transcription [11] or translation [12]. Complementary oligonucleotides have been successfully used for correction of point mutations [13] and splicing errors [14].

Recently, the phenomenon of RNA interference, the selective suppression of gene expression by double-stranded RNA [15] strictly homologous to target mRNA [16], is being actively studied. RNA interference has become a powerful tool for investigation of certain genes in a number of organisms, including mammals [17]. The ability of gene constructs to express foreign proteins provides an opportunity to correct genetic defects [18] and develop vaccines for protection against infectious diseases [19]. In a number of studies, the activation of the immune system by RNA, CpG-containing DNA [20, 21], and oligonucleotide [22, 23] as well as the influence of phosphorothioate-modified oligonucleotides on hemopoiesis, cardiac rhythm, and arterial pressure [24] have been reported. Obviously, specific interactions with target nucleic acid take place when antisense oligonucleotides or interfering RNA enter cellular compartments, whereas other effects can be caused by specific binding of nucleic acid aptamers by cell surface receptors.

Proteins play the major role in reception and transport of various molecules across biological membranes. Today one of the proteins involved in transmission of inhibitory action of double-stranded RNA from cell to cell is reliably established. By studying the mutant nematode line lacking this effect, the locus responsible for expression of SID-1 protein (necessary for systemic effect of interfering RNA [25]) was revealed. It was shown that SID-1 is a transmembrane protein carrying out transport of interfering RNA not only in *Caenorhabditis elegans* cells but also in *Drosophila* [26]. However, the latest data indicate that the systemic RNA effect requires a number of other proteins [27], and the entire transport mechanism still remains not fully understood. The role of SID-1 in transport of DNA and oligonucleotides has not been investigated.

In relation to nucleic acid transport in mammalian cells, some authors confirm that it is receptor-mediated and energetically dependent, whereas others, on the contrary, demonstrate data on energetic independence of

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nucleic acid transport. Certain ambiguity is also present when it comes to proteins mediating the transfer, as far as different scientific groups have identified different proteins. Therefore, the question about mechanism of nucleic acid transport in mammalian cells and proteins involved in this process remains open.

REVELATION OF PROTEINS INVOLVED IN BINDING AND CELLULAR UPTAKE OF NUCLEIC ACIDS

Several approaches have been applied to reveal surface cellular proteins that bind nucleic acids: native gel electrophoresis, affinity modification of proteins by phosphodiester and phosphorothioate oligonucleotide derivatives having different reactive groups, affinity chromatography, probing by oligonucleotides and DNA, as well as the use of protein-deficient animals. As a result, a variety of proteins with different molecular weights have been identified, which are assumed to participate in nucleic acid transport (table). This review attempts to at least summarize (if not to systematize) discrete information on these proteins.

Gabor and Bennett [28] were one of the first groups to report on the isolation of surface DNA-binding protein. A protein with molecular weight of 30 kD was revealed on the surface of human neutrophils by using biotinylated DNA. It was shown by using λ phage DNA labeled with ^{3}H that the protein is also present on the surface of monocytes and lymphocytes. Binding of [^{3}H]DNA with cells was saturable and could be inhibited by pre-treatment of the cells with trypsin or addition of unlabeled DNA but not RNA, poly(dA·dT), or mononucleotides. Cell treatment with pepsin, neuraminidase, phospholipase, or RNase had virtually no influence on DNA binding. With an increase in Ca^{2+}, Mg^{2+}, or SO_4^{2-} concentration in the medium up to 1 mM, the amount of cell-bound DNA also increased. The dissociation constant was 10^{-9} M for all studied cell types, and the amount of bound DNA varied between $0.81\cdot10^3$ and $2.6\cdot10^3$ molecules per cell, depending on cell type. It was demonstrated that bound DNA is transported into cells and degraded to mononucleotides. DNA transport was inhibited by addition of cycloheximide (an inhibitor of translation on 80S ribosomes) and EDTA, which confirms the need for Ca^{2+} and Mg^{2+} [29].

Hereafter the 30 kD protein isolated from membrane extract of human leukocytes by 2-D gel electrophoresis was used for immunization of BALB/c mice. After fusion of the spleen cell of immunized animal with myeloma SP/2 cells, two monoclones (which produce antibodies inhibiting DNA-binding with human leukocytes) were selected. The antibodies bound to a 30 kD protein on the cell surface specifically, showing no interaction with either histones or DNA. These antibodies were used for isolation of the 30 kD protein from human lymphocytes by affinity chromatography; it was determined that the amount of the protein on the surface of lymphocytes is $5\cdot10^5$ molecules per cell. Using flow cytometry, DNA receptor was revealed in 67% of lymphocytes and 98% of monocytes. Studying the distribution of this receptor in the population of lymphocytes by a double labeling technique, the authors demonstrated that it is expressed by 90% of B-cells and 50% of T-cells [30].

Yakubov et al. used an oligonucleotide modified with reactive alkylating group, 4-[(N-2-chloroethyl-N-methyl)-amino]benzylamine, for studies of interactions of oligonucleotides with cell surface proteins. Upon incubation of L 929 cells (mouse fibroblasts) with alkylating oligonucleotide derivative at the concentration of 0.5 μM for 30 min, proteins with molecular weights of 79 and 90 kD were modified. Additional proteins were revealed by increasing the incubation time up to 80 min, whereas nonspecific protein modification was observed at the concentration of alkylating reagent higher than 2.5 μM. The optimal concentration both for protein modification and oligonucleotide penetration into cells was 1 μM (dissociation constant is $2\cdot10^{-7}$ M), and the number of receptor molecules was estimated as $1.2\cdot10^5$ molecules per cell. The interaction of oligonucleotides with these proteins was specific since tRNA and single- and double-stranded DNA (in contrast to polyanionic heparin and chondroitin sulfates A and B) inhibited protein modification [31].

Vlasov et al. studied the interaction of alkylating derivatives of phosphodiester and phosphorothioate oligonucleotide $p(T)_{16}$ with cell lines COS-1, Vero, L-671, Ag 17-1, CHO, B7, and mouse hepatocytes. Protein doublet with molecular weight of about 79 kD and capable of oligonucleotide binding was found in all cell lines except for the hepatocytes. These proteins were absent in hepatocytes, but a protein doublet with molecular weight of ~83 kD (also found in COS-1 and Ag 17-1 cells [32]) was revealed. Later the authors applied affinity modification by oligonucleotide derivative containing photoactive *p*-azidotetrafluorobenzalhydrazone-β-alanyl and revealed 70 and 75 kD proteins in A-9 cell line. Using electron microscopy, it was shown that the derivatives of 8- and 16-mer oligonucleotide containing biotin at the 3′-end and alkylating group at the 5′-end accumulate in the nucleus [33].

Using alkylating oligonucleotide derivatives $p(T)_{10}$ and $p(T)_{15}$ at the concentration of 1 μM for affinity modification, Yakubov et al. revealed two oligonucleotide binding proteins, 79 and 35 kD, in fibroblasts from pig embryo kidney (FPEK). With increasing oligonucleotide concentration up to 2 μM an additional protein with a molecular weight of 90 kD appeared. It was shown that DNA and RNA effectively inhibit the modification of the 79 and 35 kD proteins, but not the 90 kD protein. Heparin had virtually no effect on the alkylation of all three proteins. Dextran sulfate inhibits the modification

of the 90 kD protein, has no effect on the modification of the 35 kD protein, and increases two-fold the modification of the 79 kD protein. The authors assumed that such behavior can be associated with the 79 and 90 kD proteins being subunits of the same receptor complex [34].

Oligonucleotides and modified 79 and 33 kD proteins were also revealed in the nuclei of FPEK cells, whereas the rate of nuclear accumulation was lower than the rate of cytoplasmic accumulation. Upon the replacement of culture medium with that lacking oligonucleotides, the amount of oligonucleotides bound to nuclei did not decrease (as in cytoplasm) but continued to increase, which indicated an ongoing process of oligonucleotide redistribution [35].

DNA-binding proteins with molecular weights of 46, 84, and 92 kD were revealed on the surface of HUVEC cells using [^{32}P]DNA. Proteins of 46 and 84 kD were also present in the cytoplasm and nucleus [36]. Later it was shown that monoclonal antibodies against DNA interact with 46 and 84 kD proteins on the surface of HUVEC cells, whereas treatment with DNase disrupts this interaction. Moreover, antibodies against single- and double-stranded DNA bound to 180, 110, 68, 44, 35, and 30 kD proteins, and the presence or absence of DNA had no effect on this process [37].

Loke and coworkers isolated an oligonucleotide-binding protein with molecular weight of 80 kD from HL-60 cells by chromatography on oligo(dT)-cellulose. They demonstrated that oligonucleotides are actively transported across the cell membrane, whereas the transfer efficiency is dependent on the temperature and shows a saturable pattern. According to competitive assay data, interaction with cells is mediated by the specific recognition of nucleotide (but not nucleoside and ribose). Methylphosphonate oligonucleotides did not compete with phosphodiester oligonucleotides for cell binding, which confirms the presence of specific oligonucleotide-binding proteins and proves that different oligonucleotide derivatives can be transported in cells by different pathways [38].

Gasparro et al. used 8-methoxypsoralen for photomodification of DNA bound to cell membrane and discovered three high-affinity DNA binding proteins with molecular weights of 28, 59, and 79 kD on the surface of lymphocytes [39].

Several nucleic acid binding proteins were detected by Geselowitz in promyelocytic leukemia HL-60 cells, monocytic leukemia U937 cells, and T-lymphoblastoid leukemia CEM cells using photoaffinity modification by radioactive labeled reactive oligonucleotide derivative. Incubation of the intact cells with photoactive oligonucleotide derivative revealed proteins with molecular weights of 75, 64, 52, 40, 34, and 28 kD, whereas the membrane fraction contained 75 and 28 kD proteins, and cytosolic fraction contained only insignificant amounts of 64 kD protein. Incubation with the cell nuclei revealed proteins with molecular weights of 63, 48, 38, 32, 29, and 20 kD [40].

Beltinger and coauthors used biotinylated phosphorothioate 24-mer oligonucleotide followed by treatment of the complexes with cross-linking reagent bis(sulfosuccinimidyl)suberate for affinity modification of oligonucleotide-binding proteins on the surface of human myelogenic leukemia K-562 cells. Isolation of membrane–cytosolic fraction and separation of modified proteins by electrophoresis revealed 5 main groups of proteins with molecular weights of 137-147, 79-85, 43-46, 29-32, and 20-22 kD. It was determined that the proteins do not form dimers or oligomers upon the interaction with oligonucleotide reagent; oligonucleotide binding by these proteins is specific and receptor-mediated. The authors assumed that the mentioned proteins are involved in the transport of nucleic acids in cells [41].

It was found that $G(T)_{1-7}$ oligonucleotides are able to inhibit the growth of tumor cell lines CCRF-CEM, CEM-VLB300, U937, Jurkat, H9, and HeLa. For all studied cell lines, the extent of suppression depended on the concentration and correlated with oligonucleotide binding to a nuclear protein having a molecular weight of about 45 kD [42].

Akhtar et al. studied the influence of cell differentiation on oligonucleotide penetration in Caco-2 cells (human colon adenocarcinoma). Native electrophoresis revealed a surface protein with molecular weight of 46 kD that was able to bind both phosphodiester and phosphorothioate oligonucleotides. Oligonucleotide binding to the cell surface was pH and temperature dependent [43].

The same protein with molecular weight of 46 kD was described by Hawley et al. They incubated T15 (mouse fibroblasts), COR-L23 (human lung carcinoma), and THP-1 (human monocytes) cells with 1 μM phosphorothioate oligonucleotide in Hanks solution at 4°C. Several proteins were revealed at 37°C, with the 46 kD protein being one of them. After separation of membrane–cytosolic fraction by electrophoresis followed by electroblotting and incubation with radioactive labeled phosphorothioate oligonucleotide, proteins with molecular weights of 21, 30, 35, 46, 72, 80, and 110 kD were revealed. It was experimentally established that the penetration of phosphorothioate oligonucleotide is activated by an increase in temperature; cell binding shows a saturation curve, which is competitively inhibited by single- and double-stranded DNA, i.e. the process of oligonucleotide penetration in cell displays the same main characteristics as receptor-mediated endocytosis. Moreover, oligonucleotide sequence had no effect on the set of revealed proteins [44].

Later the same authors detected major proteins with molecular weights of 28-30, 46, 67, 70-90, and 115 kD as well as a large number of minor proteins using the same experimental approach and cell lines. In this case, the set of revealed proteins depended on the cell line (T15 or

Unidentified proteins possibly implicated in transport of nucleic acid into cells

Object of study	Protein molecular weight, kD	Interacting nucleic acids	Techniques used	Reference
1	2	3	4	5
Human leukocytes (PBMC)	30	λ phage DNA	SDS-PAGE, probing by biotinylated DNA	28-30
L929	79, 90	5′-alkylating derivatives of 16-mer phosphodiester oligonucleotides	affinity modification by alkylating oligonucleotide derivatives, SDS-PAGE	31
COS-1, Vero, L-671, Ag 17-1, CHO, B7	79	5′- alkylating derivative ClR-p$(T)_{16}$	same	32
A9	75, 70	5′-photoactive derivatives of 8- and 16-mer phosphodiester oligonucleotides	– « –	33
Pig embryo kidney cells	90, 79, 35	5′-alkylating derivatives ClR-p$(T)_{10}$ and ClR-p$(T)_{15}$	– « –	34
	79, 33	5′-alkylating derivative ClR-p$(T)_{10}$		35
HUVEC	46, 84, 92	DNA	SDS-PAGE, DNA probing	36, 37
HL-60	80	p$(T)_n$	affinity chromatography on oligo-d(T)-cellulose	38
Human lymphocytes	28, 59, 79	DNA		39
HL-60, U937, CEM	75, 64, 52, 40, 34, 28	5′-photoactive derivative of 15-mer phosphodiester oligonucleotide	affinity modification by oligonucleotide derivative, SDS-PAGE	40
K562	doublets 20-22, 29-32, 43-46, 79-85, 137-147	5′-biotinylated 24-mer phosphorothioate oligonucleotide	treatment by cross-linking biotinylated oligonucleotide derivative	41
CCRF-CEM, CEM-VLB300, U937, Jurkat, H9, HeLa	45 ± 7	G$(T)_n$ ($1 \leqslant n \leqslant 7$)	affinity modification, SDS-PAGE	42
Caco-2	46	phosphodiester and phosphorothioate oligonucleotides	native gel electrophoresis	43
T15	46, 21, 30, 35, 46, 72, 80, 110	16-mer phosphodiester oligonucleotide with phosphorothioate 3′-end	native gel electrophoresis, SDS-PAGE, oligonucleotide probing	44
T15, COR-L23	28-30, 46, 67, 70-90, 115	N3′-P5′ phosphoroamide and phosphorothioate oligonucleotides	same	45
KB, HepG2, HL-60, H1	100-110	phosphodiester and phosphorothioate 21-mer oligonucleotides	affinity modification by photoactive oligonucleotide derivatives, SDS-PAGE	46

(Contd.)

1	2	3	4	5
Mouse spleen lymphocytes	18, 23, 30, 35, 40-42.5, 67-72, 78-82, 95, 145	5′-alkylating derivative of 16-mer phosphodiester oligonucleotide	affinity modification by alkylating oligonucleotide derivatives, SDS-PAGE	48, 49
HeLa, A431, HaCat	doublet 61-63, 35	5′-alkylating derivative of 16-mer phosphodiester oligonucleotide	affinity modification by alkylating oligonucleotide derivatives, SDS-PAGE	50
Human spermatocytes	~50, 30-35, <20	DNA	SDS-PAGE, DNA probing	88

COR-L23) and the oligonucleotide derivative used (N3′→ P5′ phosphoroamidates and different stereoisomers of phosphorothioate oligonucleotides, complementary to the genes *N-ras*, *C-myc*, and *IGF-1R*) [45].

Using photoaffinity modification by phosphorothioate 21-mer oligonucleotide two oligonucleotide-binding proteins having molecular weights of 100-110 kD were revealed in plasma membranes of HL-60 (human promyelocytic leukemia), HepG2 (liver carcinoma), H1 (B-cellular lymphoma), and KB (epidermal carcinoma) cells [46]. At the same time, the amount of the proteins determined by their ability to bind to oligonucleotide varied depending on the cell type. For HepG2 cells the amount of oligonucleotide-binding proteins in the case of phosphodiester oligonucleotide was $1.2 \cdot 10^6$ molecules per cell (dissociation constant was $6 \cdot 10^{-8}$ M). Phosphorothioate oligonucleotides bound to both proteins with significantly higher affinity than phosphodiester ones. The proteins were not modified after treatment of cells with trypsin, which indicated their exposure on the cell surface. Dextran sulfate, tRNA, and double-stranded DNA inhibited oligonucleotide binding with proteins, whereas ATP, ADP, AMP, and TTP had no influence on their modification [46].

Studies by Laktionov et al. demonstrated that oligonucleotide with no mitogenic effect competitively inhibit the mitogenic effect of plasmid DNA on mouse spleen lymphocytes. Using an alkylating derivative of 16-mer oligonucleotide (4-[(N-2-chloroethyl-N-methyl)-amino]benzylamine-p$(N)_{16}$), the authors detected DNA-binding proteins on the cell surface with molecular weights of 15, 18, 40, 42.5, 67, 72, 77, and 82 kD. The highest affinity towards oligonucleotides was displayed by the 42.5 kD protein (dissociation constant for its oligonucleotide complex was $5 \cdot 10^{-7}$ M) [47-49].

Oligonucleotide-binding proteins with molecular weights of 61-63 and 35 kD were revealed in keratinocytes using the same alkylating oligonucleotide [50]. As was determined by cell fractionation, the proteins are mainly localized on the plasma membrane, but can also be found in nuclear fraction. Pretreatment with trypsin and preincubation of A431 cells with polyanions (heparin, dextran sulfate) or with single- and double-stranded DNA inhibited the interaction between oligonucleotide-binding proteins and reactive oligonucleotide derivative, whereas an excess of ATP in the medium had no such effect. The presence of the proteins both on the plasma membrane and in cell nucleus as well as suppression of affinity modification by trypsin suggests that the proteins are involved in recognition of oligonucleotides on the cell surface, and their transport across plasma membrane into the cell is followed by their penetration into the nucleus [51].

Later this work was continued using cells of different origin: A431, HeLa, KB, MCF-7, Hep-2, human embryo lung (HEL), K562, Cos-7, FPEK, and NIH/3T3. It was demonstrated that the same set of cell surface oligonucleotide-binding proteins is characteristic for all studied cell lines. Upon incubation of the cells with oligonucleotide derivatives in phosphate buffer (without Ca^{2+} and Mg^{2+}), proteins with molecular weights of 68, 38, and 28 kD were revealed, whereas upon incubation in the culture medium an additional 46 kD protein was found and the extent of modification for 68, 38, and 28 kD proteins was increased [52, 53]. It should be noted that the 68 and 38 kD proteins correspond to 61-63 and 35 kD proteins [50, 51] revealed earlier, and possibly to 79 and 33(35) kD proteins [34, 35], taking into account the potential error in determination of protein molecular weight.

A protein with molecular weight of 38 kD was isolated by affinity chromatography on Ultragel A2 with immobilized oligonucleotide pCAGTAAATATCTAGGA and identified as glyceraldehyde-3-phosphate dehydrogenase [54]. The method developed by Laktionov et al. [55, 56] was used for the isolation of a 68 kD protein. The method is based on affinity modification of proteins in intact cells by oligonucleotide derivative Flu-Lys-p$(N)_{16}$-deg-U containing a reactive group at one end (oxidized

rU coupled through diethyl glycol linker (deg)) and a hapten at the other end (fluorescein, Flu), followed by isolation of oligonucleotide–protein complexes by affinity chromatography on Ultragel A2 with immobilized anti-fluorescein antibodies. It appeared that the 68 kD protein band contains four proteins with very similar molecular weights: albumin, keratin K1, keratin K10, and keratin K2e.

Thus, a great number of proteins with different molecular weights have been revealed, which are probably involved in nucleic acid transport. It is likely that some of them correspond to already known proteins, while others are to be identified.

IDENTIFICATION OF PROTEINS INVOLVED IN BINDING AND UPTAKE OF NUCLEIC ACIDS

Glyceraldehyde-3-phosphate dehydrogenase. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was for a long time considered as a classical glycolytic enzyme. However, it has been shown in recent years that GAPDH is also implicated in RNA export from the nucleus [57] and displays phosphotransferase [58] and uracyl-DNA-glycosidase activity [59, 60]. Some researchers also suppose that the enzyme takes part in a variety of processes associated with apoptosis, age-related neurodegenerative diseases, and prostate cancer.

RNA-binding function of GAPDH was discovered by affinity chromatography of lysate of rabbit reticulocytes (in which the ribosomal proteins were depleted) on rRNA-Sepharose. Isolated protein with molecular weight of 36 kD was identified as glyceraldehyde-3-phosphate dehydrogenase [61]. It was determined that GAPDH specifically interacts with 3′- and 5′-untranslatable regions of mRNA, and the RNA binding site is located in an NAD^+-binding domain [62]. Specific binding of GAPDH with 3′- untranslatable region of papilloma virus RNA [63] and 5′-untranslatable region of hepatitis A virus RNA [64] was also reported.

A number of facts indicate that GAPDH can be implicated in RNA transport. Thus, under the study of human $tRNA^{Met}$ transport from frog oocyte nucleus, a dramatic decrease in export was found for tRNA containing a single base replacement in the TΨC-loop. To investigate the mechanism of such selective transport, nuclear extract of HeLa cells was analyzed with the aim of detecting the proteins that can change the electrophoretic mobility of wild type $tRNA^{Met}$ and at the same time have no effect on the mobility of mutant molecules (under conditions of native electrophoresis). Such a protein was indeed revealed. Its molecular weight estimated by electrophoretic mobility was 37 kD, and the analysis of C-terminal sequence confirmed that the isolated protein is GAPDH. Using a different mutant tRNA, it was found that the main recognition site for GAPDH is the TΨC-loop; however, replacements in other tRNA regions also influence its interaction with GAPDH.

Study of the competitive effect of NAD^+ in the concentration range 0.02-50 μM has shown that the interaction between GAPDH and tRNA is completely inhibited at the concentration of 5 μM, which corresponds to earlier data on the location of RNA-binding site in the NAD^+-binding domain. According to the authors' assumption, such an interaction in the nucleus can have a regulatory role in tRNA transport [57]. It is probable that GAPDH is also implicated in RNA transport in the cell, since the enzyme is present on plasma membrane as well. Thus, using affinity chromatography on Sepharose 4B with immobilized yeast tRNA from membrane extract, two RNA-binding proteins were isolated, having molecular weights of 37 and 45 kD. The proteins were separated by gel filtration and sequenced. It turned out that the 37 kD protein is glyceraldehyde-3-phosphate dehydrogenase, and the 45 kD protein is 3-phosphoglycerate kinase 1 [65].

GAPDH can interact not only with RNA but also with DNA. Grifoni et al. found a protein with molecular weight of 38 kD that is capable of sequence-specific interaction with oligonucleotides comprising TAAAT sequence, using native electrophoresis for separation of HeLa nuclear extract. Phosphorothioate analogs of the oligonucleotide, complementary to the translation initiation site of the cytoplasmic phospholipase A2 gene (which contains TAAAT sequence), are accumulated in compact structures in HeLa nuclei, whereas phosphorothioate oligonucleotides complementary to cyclooxygenase-2 gene sequence are localized entirely in the cytoplasm [66].

Later a 38 kD protein was isolated from nuclear extract of HeLa cells by affinity chromatography. Its amino acid sequence has been determined, and it was proved that the protein is GAPDH [54]. The oligonucleotide-binding region was localized between amino acid residues 286 and 334 in the C-terminal fragment of the GAPDH molecule. This region belongs both to the NAD^+ and glyceraldehyde-3-phosphate binding domains of the enzyme, and at the same time it is distant from the glyceraldehyde-3-phosphate and NAD^+ binding sites. Hence, the oligonucleotide-binding site is apparently a distinct domain within the GAPDH molecule. Since the GAPDH sites of nuclear localization (259-263) and nuclear export (202-208) do not overlap with the oligonucleotide-binding site, it can be assumed that the oligonucleotide–protein complex formed is transported into the cell nucleus [67, 68]. Therefore, it seems that GAPDH can be involved in oligonucleotide transport from the cell surface into the cell nucleus.

Albumin. Geselowitz and Neckers have demonstrated that upon treatment of HL-60 cells cultured in medium containing standard bovine serum albumin (BSA) with photoactive oligonucleotide derivative, virtually all

cell-bound radioactive labeled oligonucleotide was associated with one protein, which was identified as BSA. Maximal protein modification was observed in the case when the cells were cultured in BSA-containing medium, and then incubated with oligonucleotide derivative in the absence of BSA. Modification of cell-bound protein was significantly lower upon cell incubation in the medium with BSA; in this case, the presence or absence of BSA during preliminary cell cultivation had no effect on its modification. If BSA was absent during both cell cultivation and incubation with oligonucleotide derivative, modified protein was not detected at all. Based on these results the authors supposed that BSA completely blocks oligonucleotide interaction with cells and is the major cell-associated oligonucleotide-binding protein [69].

Using the method developed by Laktionov et al., albumin was isolated from membrane–cytosolic fraction of A431 cells within a group of proteins with molecular weight of 68 kD [55, 56].

A number of studies illustrate high affinity interaction between nucleic acids and albumin. Thus, the dissociation constant for the complex of 16-mer phosphodiester oligonucleotide with human serum albumin (HSA) was $2 \cdot 10^{-5}$ M. Double-stranded DNA competitively inhibited oligonucleotide binding to HSA, more effectively than added oligonucleotide competitors. This suggested that double-stranded DNA more effectively binds to HSA than single-stranded oligonucleotides [70, 71].

The dissociation constants for phosphorothioate oligonucleotides varied between $2.4 \cdot 10^{-4}$ and $4.8 \cdot 10^{-5}$ M. Apparently, binding of phosphorothioate oligonucleotide takes place in site I, since it was inhibited by warfarin (typical site I ligand). The best binding was observed for oligonucleotide derivative containing cholesterol at the 5′-end [72].

Human serum albumin comprises three homologous domains (I-III), each of those being divided into two subdomains (A and B). Sub-domains A and B have six and four α-helices, respectively, and are connected by flexible loops [73]. HSA has two drug binding sites (I and II), located in sub-domains IIA and IIIA, respectively [74]. The two sites have similar structure and represent hydrophobic pockets surrounded by positively charged residues. Site I is significantly larger than site II and is able to bind such a large ligand as bilirubin. Moreover, there are data on independent binding of two ligands with this site [75]. Considering the structure of site I, it is easy to explain its interaction with phosphorothioate oligonucleotides containing the cholesterol moiety.

It is possible that one more binding site can exist in the albumin molecule for binding of phosphodiester oligonucleotides that do not contain hydrophobic groups. Thus, upon the study of HSA interaction with 27-mer fluorescein-labeled oligonucleotides, an assumption was made about the existence of an additional oligonucleotide-binding site [72]. Indeed, computer analysis of serum albumin structure revealed a potential RNA and DNA binding site in sub-domain IIIB (region 570-584) [76]. On the other hand, according to the data of Smidt and coauthors, phosphodiester oligonucleotides ($p(T)_{16}$) containing cholesterol at the 5′-end bind to high and low density blood lipoproteins, but do not bind to albumin [77], which is contradictory to the data presented by other researchers [69-72, 76].

It is likely that albumin, not being a membrane protein, interacts with cell surface through exposed albumin receptors [78, 79]. In addition, albumin can improve oligonucleotide transport in cells, and in nuclei in particular [80]. In this connection, it may be possible to use albumin as a delivery agent. Indeed, the method for delivery of oligonucleotides (as components of nanoparticles) in cells has been suggested [81-83].

Proteins responsible for nucleic acid transport in spermatozoids. Wu et al. found major histocompatibility complex II (MHC II) on the surface of mouse spermatozoids and demonstrated that it is involved in binding of exogenic DNA [84]. Another potential nucleic acid receptor in spermatozoids is CD4, which is known to be exposed on the cell surface. Hence, Yakubov and coworkers have determined that recombinant CD4 is able to bind phosphorothioate and phosphodiester oligonucleotides in solution. Two sites for oligonucleotide binding with different affinity (10^{-7} and 10^{-6} M) were revealed in the CD4 molecule [85].

Further studies demonstrated that spermatozoids of MHC II-deficient mice bind DNA to a lower extent than spermatozoids of wild type mice, while spermatozoids of CD4-deficient mice display the same level of DNA binding as wild type cells, but they cannot take it up. Moreover, wild type spermatozoids lost their ability to uptake DNA after incubation with anti-CD4 antibodies. Using the antibodies, the authors detected CD4 at the head of the spermatozoid, but could not detect MHC II, and therefore concluded that MHC II is required during spermatogenesis for formation of spermatozoids able to bind DNA [86].

During the study of nucleic acid transport in spermatozoids, it was found that plasmid DNA is quickly taken up (during 15-20 min) and is accumulated in the nuclear region. DNA uptake was inhibited by addition of heparin, dextran sulfate, and excess of non-labeled DNA, whereas poly-L-lysine, on the contrary, facilitated the penetration. Proteins with molecular weights of 30-35 kD were revealed, which are possibly involved in binding of exogenic DNA [87]. Later the same authors revealed three classes of DNA-binding proteins in the extract of spermatozoid head: proteins with molecular weights below 20, 30-35, and about 50 kD. However, according to the authors' point of view, only the 30-35 kD proteins are involved in binding of exogenic DNA [88].

Later it was found that only mature spermatozoids are able to bind and uptake DNA, although the mecha-

nism for DNA transport from the cytoplasm into the nucleus is also functional in immature spermatozoids, as was shown by ultrasonic DNA introduction. Based on these results, an assumption was made that 30-35 kD proteins are implicated in DNA binding and internalization, but they also can be components of the cytosolic transport system. Other DNA-binding proteins were also suggested to function as DNA receptors, among those being 60 and 100 kD proteins since they are present in mature spermatozoids and practically absent in immature ones. The authors could not detect MHC II in spermatozoids; the CD4 level was low and did not depend on the degree of spermatozoid maturation [89]. Therefore, MHC II and CD4 are unlikely to take part in nucleic acid transport. The 60 and 100 kD proteins, putative transporters of nucleic acids, still remain unknown. Taking into account the error in determination of molecular weight, one can assume that it is nucleolin.

Nucleolin. Ishikawa and coauthors isolated nuclear proteins, which specifically interact with DNA telomere repeat $d(TTAGGG)_n$, from HeLa cells. Sequencing and immunoassay results indicated that the isolated proteins are identical or similar to heterogeneous nuclear ribonucleoproteins A1, A2-B1, D, and E, and nucleolin [90]. A protein with molecular weight of 110 kD, binding to phosphorothioate oligonucleotides, was found by Weidner et al. in the nuclear extract of LOX cells (amelanotic melanoma) and identified as C23/nucleolin [91].

Later a oligonucleotide-binding protein with molecular weight of 106 kD was revealed by Bates and coauthors in nuclear extract of HeLa cells [92]. It was found that an anti-proliferating effect of oligonucleotides correlates with their binding by this protein: G-rich oligonucleotides with high anti-proliferating activity were effectively bound, while oligonucleotides with low or no activity either weakly interacted with the protein or did not interact at all. Based on electrophoresis and immunoblotting data, the assumption was made that the protein is nucleolin.

To reveal oligonucleotide-binding proteins, MDA-MB-231 cells were incubated with biotinylated derivatives of active (displaying high anti-proliferating activity) and inactive oligonucleotides, then oligonucleotide–protein complexes were isolated from cell lysates using magnetic particles coated with streptavidin. A 116 kD protein was bound to both active and inactive oligonucleotides, but in the latter case the extent of binding was noticeably lower. It was demonstrated that the protein also interacts with antibodies against nucleolin. To determine the localization of the protein, the membrane, cytosolic, and nuclear fractions were separated by electrophoresis and protein fractions were analyzed by their ability to interact with anti-nucleolin antiserum and radioactively labeled oligonucleotides. The 116 kD protein was revealed in nuclear and, to a lesser extent, in cytosolic fractions, as well as on the cell membrane. In addition, a protein of 70 kD was detected in all fractions, which interacted both with oligonucleotides and antibodies against nucleolin. It seems that the protein is a proteolytic fragment of nucleolin [92].

Ezrin and moesin. Tschakarjan et al. isolated two DNA-binding proteins with molecular weights of 81 and 77 kD from lysate of human mammary gland epidermis by 2-D gel electrophoresis. Using mass spectrometry, the proteins were identified as ezrin and moesin. Since these proteins are localized on the inner surface of plasma membrane, it is supposed that nucleic acid transport requires their interaction with cell surface receptor [93].

Keratins. For a long time it was believed that keratins belonging to the intermediate filament protein family are only involved in formation of cytoskeleton. However, recent studies have led to reconsideration of this point of view. Thus, it was demonstrated that keratin K8 is a plasminogen receptor in endothelial cells, hepatocytes, and mammary gland cancer cells [94-97], and keratin K18 binds to thrombin–antithrombin complex [98]. Moreover, it was found that keratin K18 interacts with DNA [99], and keratins K1, K10, and K2e were isolated from the surface of A431 cell line by affinity chromatography as proteins carrying out the reception and transport of nucleic acids [55, 56, 100].

As has been shown in a number of investigations, keratin K1 is exposed on the outer cell surface. Mahdi and coauthors used specific antibodies against epitopes encoded by the 1st and 2nd exons of the keratin K1 gene for determination of cellular localization of this protein. Using these antibodies for immunostaining with horseradish peroxidase, immunofluorescence, and transmission microscopy, it was established that at least these two epitopes of keratin K1 are exposed on the outer surface of intact endothelial cells from human umbilical vein (HUVEC). The presence of keratin K1 on the surface of HUVEC cells was confirmed by flow cytometry. Besides, the number of keratin K1 molecules exposed on the cell surface was determined, which was equal to $7.2 \cdot 10^4$ molecules per cell [101]. These data are in agreement with the amount of oligonucleotide-binding protein with molecular weight of 68 kD on the surface of A431 epithelial cells ($2.5 \cdot 10^5$ binding sites per cell) [51].

Recently it was established that keratin K1 is localized on the endothelial cell membrane together with urokinase-type plasminogen activation receptor (uPAR) [101] and thus is engaged in a multi-protein receptor complex. Being a component of this complex, keratin K1 is exposed on the outer cell surface of HUVEC cells and is involved in binding of kininogen [102] and factor XII [103]. It was illustrated that keratin K1 is implicated in the regulation of activity of the kallikrein–kinin system [104], and its ability to be phosphorylated by cellular kinases suggests its role in the induction of intracellular signaling pathways [105].

A crucial event in the activation of the kallikrein–kinin system is the conversion of pre-

kallikrein into kallikrein. Such a conversion occurs on the surface of endothelial cells according to a not completely understood mechanism, and only in the case if high molecular weight kininogens are also bound to endothelial cells. It was demonstrated that antibodies against keratin K1 block pre-kallikrein binding to cells and thus prevent its activation [101]. Therefore, it can be supposed that pre-kallikrein binding to keratin K1 is necessary for normal functioning of the kallikrein–kinin system.

It is known that phosphorothioate oligonucleotides, displaying enhanced affinity to proteins, affect arterial pressure, cardiac emission, and beat frequency [106]. One of the mechanisms explaining such effects of nucleic acids can be associated with competitive binding to keratin K1, which results in the inhibition of the kallikrein–kinin system.

Membrane channel proteins. To reveal oligonucleotide-binding proteins on the outer membrane of the cells covering the tubules in rat kidney, Hanss and coworkers applied an affinity chromatography. Using heterogeneous biotinylated 20-mer phosphorothioate oligonucleotide bio-d(TCCCAGGCTCAGATCTGGTC) immobilized on streptavidin-agarose, they isolated a protein with molecular weight of 45 kD and characterized it as a membrane channel transporting short single-stranded nucleic acid sequences [107]. It was demonstrated in experiments with artificial lipid membrane that the ability of this channel to transport oligonucleotides depends on the concentration of calcium ions: carrying capacity in the presence of 1 mM Ca^{2+} was 100 times higher than in the absence of calcium [108]. In addition, it was determined that such ions as K^+, Cs^+, and Cl^- cannot be transported through this channel, which indicates, according to authors' opinion, its high specificity towards nucleic acids. Oligonucleotide transfer was significantly hampered upon addition of heparin sulfate, and completely suppressed after heat inactivation of the protein. Phosphorothioate and phosphodiester oligonucleotides had no difference in their ability to be transported through this channel [109].

Afterwards the authors modified the procedure for protein isolation and found that the discovered membrane channel is a multi-protein complex, which contains the channel itself, formed by a protein with molecular weight of 45 kD (p45), and transporting nucleic acids (NACh), and a regulatory protein with a molecular weight of 36 kD (p36). Both proteins were purified by 2-D gel electrophoresis and sequenced by mass spectrometry. The 36 kD protein was identified as cytosolic rat malate dehydrogenase (cMDH), while the sequence of p45 unfortunately could not be found in the database [110].

Transcription factors can also act as channels for transport of nucleic acids across lipid membrane. As was shown in experiments with artificial lipid membrane, transcription factors ADD1, GCN4, NF-AT4, and Fos-Jun are able to form ion channels, which recognize double-stranded DNA containing consensus sequences. Active channels were formed upon addition of transcription factors and DNA from the opposite sides of the lipid membrane, and if the proteins contained dimerization domains. Incorporation of mutations in dimerizing domains of transcription factors ADD1 and GCN4 prevented channel formation. The same effect was observed after separate addition of factors Fos and Jun to the lipid membrane, since they can form only heterodimers but not homodimers. Conductivity of the channels formed by different factors varied. Addition of DNA, which did not contain the recognition sequence for a corresponding factor, did not result in the change of channel conductivity, thus indicating their strict sequence specificity [111]. Nevertheless, all the experiments were carried out in the artificial system, and therefore it is not clear if the above factors are involved in nucleic acid transport in a real cell.

Membrane protein MNAB. Siess et al. cloned the gene of a surface DNA-binding protein with a molecular weight of about 130 kD. The sequence contained 4351 bp with an open reading frame of 3576 bp, coding for a protein of 1192 amino acid residues. The gene was found by screening of the human monocyte cDNA library using vector λgt 11 and serum of systemic lupus erythematosus patients, which has an ability to competitively inhibit DNA binding to cell surface by interacting with DNA-binding proteins. It was determined that the gene is located on chromosome 9. A protein with molecular weight of 150 kD was isolated from S49 cells by using biotinylated DNA. The protein interacted with the serum of systemic lupus erythematosus patients and rabbit polyclonal antibodies against the cloned protein. Therefore, the conclusion was made that the discovered gene encodes for a DNA-binding protein, MNAB.

Analysis of protein structure revealed several functional domains such as a zinc finger localized approximately in the center of the protein molecule (amino acid residues 416-435), a RING finger (amino acid residues 14-50), and possible transmembrane domain in the vicinity of the carboxyl end (amino acid residues 1133-1171). It was shown that the binding constant of MNAB to DNA is approximately $4 \cdot 10^{-9}$ M, while a point mutation in the conservative region of the zinc finger decreases this value by 50%. It was determined by cell fractionation that practically all MNAB protein is associated with membrane fraction. Using fluorescent microscopy the protein was localized mainly in the perinuclear space, but not in the nucleus or on the cell surface (probably, due to insufficient sensitivity of the method). Nevertheless, the authors localized MNAB on the cell surface using flow cytometry [112].

Heparin-binding integrin Mac-1. Mac-1 represents a heparin-binding integrin located on the surface of leukocytes, monocytes, macrophages, and natural killers; it is involved in the process of leukocyte adhesion and migration, and it mediates binding of polymorphic nuclear leukocytes with endothelial cells and extracellular matrix.

Benimetscaya and coworkers studied the interaction of phosphodiester and phosphorothioate oligonucleotides with Mac-1 and found that the stimulation of expression of this protein is accompanied by an increase in oligonucleotide binding to the cell surface and its internalization. The authors supposed that Mac-1 is a surface cellular receptor for oligonucleotides, which mediates their penetration into cells. Soluble fibrinogen (a natural ligand for Mac-1) and monoclonal antibodies against Mac-1 substantially inhibited oligonucleotide binding with activated and non-activated polymorphic nuclear leukocytes and HL-60 cells. As was shown by using alkylating derivative of phosphodiester oligonucleotide, oligonucleotides bind to both Mac-1 subunits, 95 kD (β2) and 195 kD (αM), with dissociation constants of $8.8 \cdot 10^{-6}$ and $1.7 \cdot 10^{-5}$ M, respectively. The presence of Ca^{2+} or Mg^{2+} was required for binding of phosphodiester oligonucleotides with Mac-1, whereas phosphorothioate oligonucleotides were able to interact with Mac-1 independently of the presence of these cations [113].

Proteins involved in the immunostimulating effect of CpG-containing DNA. A number of researcher have demonstrated that bacterial DNA has a stimulating effect on the mammalian immune system, where the effect is due to the presence of unmethylated cytosines in CpG-sequences. Synthetic oligonucleotides comprising these sequences as well as CpG-containing DNA are able to induce proliferation of B-lymphocytes and production of cytokines in different immune cells [114-116].

Studies of proteins involved in the immunostimulating effect of CpG-DNA were mainly carried out using mouse lines deficient in different proteins. For example, it was shown that activation of the TLR/IL-1R (TLR stems from toll-like receptor) signaling pathway by CpG-DNA is mediated by a differentiation marker of myeloid cells (MyD88) and by tumor necrosis factor receptor-associated factor (TRAF6) [117]. The comparison of wild type mice and mice deficient in DNA-dependent protein kinase revealed that DNA-dependent protein kinase is necessary for the immunostimulating effect of oligonucleotides and bacterial DNA [118]. On the other hand, according to Hemmi et al., CpG-DNA has the same immunostimulating effect on both wild type mouse dendritic cells and DNA-dependent protein kinase-deficient cells, whereas no immune response towards CpG-DNA was observed for MyD88 and TLR9-deficient cells [119]. Indeed, it was demonstrated that the level of TLR9 expression correlated with the cell ability to react against CpG-DNA [120]. Since CpG-DNA had no immunostimulating effect in TLR9-deficient mice, Hemmi et al. concluded that the immunostimulating effect of CpG-DNA requires its interaction with TLR9 [121].

According to Wagner's data, CpG-containing DNA is recognized by endosomes after nonspecific cellular uptake [122]. It remains unclear where the interaction between TLR9 and DNA takes place, either on the cell surface or in endosomes. It was shown by flow cytometry that TLR1, TLR2, and TLR4 are exposed on the cell surface [123-126], and this observation suggests similar localization for TLR9 as well. It is known that TLR2 can relocate from cell surface to phagosome after stimulation with zymosan [127, 128], and thus it can recognize the ligand both exposed on the cell surface and in the phagosome after its cleavage. Apparently, TLR9 can act in a similar way.

Thus, it is possible that the role of TLR9 in the immunostimulating effect is concluded in binding of CpG-DNA on the cell surface, but there is no direct evidence for this assumption.

Scavenger receptor. Kimura and coworkers found that oligonucleotides displaying immunostimulating effect interfere with the interaction of acetylated lipoprotein with scavenger receptor of mouse splenocytes. Addition of dextran sulfate and polyvinyl sulfate inhibited the activation of splenocytes by oligonucleotides. The assumption was made that immunostimulating effect of oligonucleotides requires their binding by scavenger receptor [129].

Later a number of studies were published where the interaction of scavenger receptors with nucleic acids was confirmed. Thus, it was shown that the injection of rats with antisense phosphorothioate oligonucleotides results in the major amount of radioactive material being quickly accumulated in liver, bone marrow, and kidneys (the time of oligonucleotide half removal from the bloodstream is about 24 min). The oligonucleotide distribution in liver tissue strictly corresponded to that of acetylated low density lipoproteins (classical ligands for scavenger receptors A, types I and II), and the maximal accumulation was observed in endothelial cells. To verify the hypothesis on the participation of scavenger receptor class A types I and II in oligonucleotide binding, the rats were injected with poly(I) and poly(A) [130]. As was determined earlier, poly(I) and poly(G) are the ligands for scavenger receptor, contrary to poly(C) and poly(A), which have different tertiary structure [131]. However, it appeared that the tertiary structure is important only for the formation of oligonucleotide aggregates, which properly bind to the receptor. Oligonucleotides, which do not form aggregates, cannot interact with scavenger receptor independently of their structure [132].

Study of pharmacokinetics of phosphorothioate oligonucleotides in rats in the presence of poly(I) and poly(A) has demonstrated that poly(I) substantially reduces the removal of oligonucleotides from the bloodstream, whereas poly(A) has no measurable effect on the process. Accumulation of oligonucleotides in liver and bone marrow was saturable and inhibited by poly(I) but not poly(A), which indicated the involvement of scavenger receptor (types AI and AII) in oligonucleotide binding and internalization by these organs. In contrast,

the oligonucleotide accumulation in kidneys was inhibited by poly(A) and not poly(I), which can be explained by the presence of another type of scavenger receptors. Oligonucleotide uptake by spleen, muscles, and skin was insignificant, showed weak dose dependency, and was not inhibited by either poly(A) or poly(I) [130].

Similar results were obtained by Steward and coauthors. Introduction of known scavenger receptor ligands in mice (poly(I), dextran sulfate, fucoidan) significantly diminished the accumulation of phosphorothioate oligonucleotide in liver and spleen, and slightly increased that in skeletal muscles. Polyanions, which are not substrates for scavenger receptor (poly(C) and chondroitin sulfate), did not influence the tissue distribution of oligonucleotides in rats. Introduction of dextran sulfate also decreased accumulation of phosphorothioate oligonucleotides in liver and increased their accumulation in skeletal muscles. Oligonucleotide binding in kidneys was decreased; however, oligonucleotide excretion in urine was increased, which was caused by the decrease in its re-adsorption in proximal tubules. Also, the addition of dextran sulfate and fucoidan substantially decreased the accumulation of phosphorothioate oligonucleotides by J774 cells (mouse macrophages), whereas chondroitin sulfate had no effect on oligonucleotide binding to cells [133].

Studies performed in recent years have led researchers to reconsider the role of scavenger receptors in binding and transport of nucleic acids. Thus, Benimetscaya et al. showed that such scavenger receptor ligands as oxidized and acetylated lipoproteins and methylated BSA have no effect on binding of fluorescein labeled phosphorothioate oligonucleotide $(C)_{28}$ with macrophages and human microglial cells [113]. According to the data of Takura and coworkers, uptake of plasmid DNA by cultured Chinese hamster oocytes (CHO) expressing scavenger receptor A (SRA) is insignificant and practically identical to that of wild type CHO cells, and macrophages of SRA-deficient mice are able to bind and uptake plasmid DNA to the same extent as macrophages of control mice. Binding and uptake of plasmid DNA was inhibited by non-labeled DNA, poly(I), and dextran sulfate (but not by poly(A) and acetylated lipoproteins) in both types of macrophages. According to authors' opinion, these data indicate that the mechanism for binding and uptake of plasmid DNA by peritoneal macrophages is similar to that for polyanion binding to scavenger receptor, but the scavenger receptor itself is not involved in the process [134].

Butler and coauthors showed similar results by comparison of wild type and SRA-deficient mice. It was demonstrated that intravenous introduction of phosphorothioate oligonucleotides results in their identical accumulation and distribution patterns in tissues and cells of both mouse types. Moreover, antisense oligonucleotides against A-*raf* gene diminished the level of A-*raf* mRNA to the same extent. Similar data were obtained in peritoneal macrophages [135].

Bacterial DNA and CpG-oligonucleotides stimulated the production of IL-12 by wild type and SRA-deficient mouse macrophages, whereas the oligonucleotides lacking CpG-sequences and DNA from calf thymus did not have the immunostimulating effect. It was shown by confocal microscopy that fluorescein labeled oligonucleotides were accumulated in the cytoplasm and colocalized with dextran sulfate, which was transported into cells via a pinocytosis mechanism. The level of oligonucleotide accumulation and their distribution pattern were identical for both macrophage types. These data suggest that SRA, although is able to bind DNA, is not important for uptake of CpG-containing oligonucleotides and bacterial DNA, as well as for their immunostimulating properties [136].

It should be pointed out that the story of a scavenger receptor is not the only case when the original discovery was not confirmed by later studies. It is rather the rule in the area of investigation of proteins involved in reception and transport of nucleic acid into cells. Thus, de Diesbach and coworkers isolated a cellular protein with a molecular weight of 66 kD from HepG2 hepatocytes (human liver carcinoma) using photoaffinity modification by biotinylated oligonucleotide conjugate with benzophenone and subsequent affinity chromatography. It was shown that oligonucleotide binding with the isolated protein is saturable and competitively inhibited by non-labeled DNA. Virtually all modified 66 kD protein was in a membrane fraction. About half of the protein bound to oligonucleotide underwent surface proteolysis, which suggests the localization of the protein both on the cell surface and in vesicular cytoplasmic structures. Sequencing of the isolated protein by the Edman method did not reveal any homology with known human proteins [137]. Subsequently, the authors determined that oligonucleotides penetrate into HepG2 cells by two different mechanisms and are accumulated in endosome-like structures of two types, which are significantly different from normal endosomes and lysosomes [138], which was contradictory to the results of other studies. Later, a simple explanation was found for these data: HepG2 cell line used throughout the experiments was infected with *Mycoplasma hyorhinis*, which, as turned out, altered the pattern of oligonucleotide penetration into cells. Moreover, the 66 kD protein, previously isolated from the HepG2 cell line as a membrane receptor for nucleic acids, appeared to be the invariant membrane protein from *M. hyorhinis* [139].

Thereby, differences in experimental models, oligonucleotide sequences, reactive groups and their coupling to oligonucleotides, and modification conditions, as well as errors in determination of protein molecular weights and possible use of contaminated cultures, resulted in the revelation of a large number of putative proteins

involved in binding and transport of nucleic acids. Despite the fact that some of these proteins have been identified and possible transport mechanisms described, today there is no complete picture of nucleic acid transport across the cell membrane.

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