Hochschule Aachen

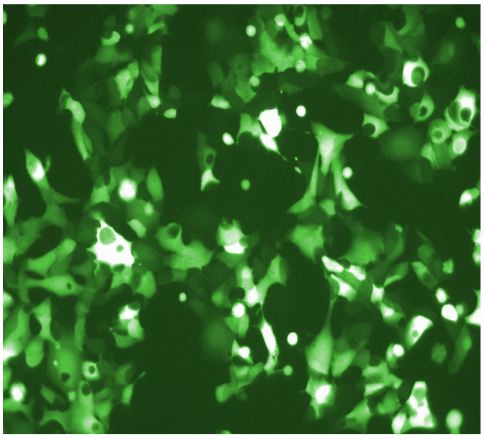
Fachhochschule Aachen, Abteilung Jülich

Fachbereich 12, Bioingenieurwesen, Biotechnologische Verfahren

Diplomarbeit

Thema:

Development of a gene filter array protocol for studies of gene regulation by AMP activated protein kinase (AMPK)



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Stockholm, Schweden, Mai 2002

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Diese Diplomarbeit ist von David Krämer selbständig abgefertigt und verfasst.
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David Krämer, Mai 2002

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Diese Arbeit wurde in Kooperation mit der Firma Biovitrum AB (Strandbergsg. 49, 112 76 Stockholm) und der Fakultät für Physiologie und Pharmakologie des Karolinska Institutet (Solna, Stockholm) in Schweden durchgeführt.

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Last but not least I must not forget Jan Hörling who established the first contact, even though I could have been a moron…

God could cause us considerable embarrassment by revealing all the secrets of nature to us: we should not know what to do for sheer apathy and boredom.
Johann Wolfgang von Goethe (1749-1832), German poet and dramatist.

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Abbreviations

ACC	acetyl-CoA carboxylase
Ad	Adenovirus
AICAR	5-aminoimidazole-4-carboxamide-riboside
AMPK	AMP activated protein kinase
AMPKK	AMPK kinase
CAR	Cellular adenoviral receptor
CBP	creb binding protein
CDS	coding regions
CMV	cytomgalovirus
CPT	carnitine palmitoyltransferase
ERK	extracellular signal-regulated kinase5
FAS	fatty acid synthase
FBS	foetal bovine serum
G3PDK	glycerin-3-phosphate dehydrogenase
GFP	green fluorescent protein
GLUT	glucose transporter
GPAT	sn-glycerol-3-phosphate acyltransferase
GRB	growth-factor receptor bound protein
GSK	glycogen synthase kinase
HSL	hormone sensitive lipase
IDDM	Insulin-dependent (type I) diabetes mellitus
IRS	insulin receptor substrate
MEF	myocyte enhancer factor
MOI	Multiple of infection
NIDDM	non-insulin-dependent (type II) diabetes mellitus
o/n	over night
p38MAPK	p38 mitogen-activated protein kinase
PEPCK	phosphoenolpyruvate carboxykinase
PFU	plaque forming units
PI3K	phosphatidylinositol 3-kinase
PKA	protein kinase A
PKB	protein kinase B
PP1	protein phosphatase 1
PP2C	putative protein phosphatase
PP5	protein phosphatase 5
PTP1B	protein tyrosine phosphatase 1b
sscDNA	single stranded copy DNA
STAT5B	signal transducer and activator of transcription 5b
TLC	thin layer chromatography
UCP	uncoupling protein

Abstract

The aim of this study was to produce DNA filter arrays and develop a protocol to investigate the impact of AMP activated protein kinase (AMPK) on gene expression in primary human myocytes. 67 candidate genes were selected. Some of them had already been described as regulated by AMPK, or by a synthetic AMP analogue (5-aminoimidazole-4-carboxamide-riboside, (AICAR)) which is known as an AMPK activator. Many of the selected genes are believed to be part of pathways AMPK is involved in. Another aspect of AMPK, the acute regulation of enzymes or other proteins by phosphorylation has not been addressed in the present study.

Human primary myocytes were infected with a recombinant adenovirus, carrying the human AMPKα_2-gene. Using PCR technique oligo-nucleotides of an approximate length of 150 bp were produced from the chosen genes. They were then fixed on nylon material filters and hybridised with P^{33}-labelled cDNA that was obtained from mRNA, which had been isolated from the AMPKα_2-infected cells.

The AMPKα_2-gene containing adenovirus had been constructed at Biovitrum before[1]. The gene was overexpressed under the control of a strong promoter (CMV promoter)[2]. AMPK activity requires co-operation of α, β and γ subunits[8]. In skeletal muscle α_2, β_2, γ_2 and γ_3 subunits are endogenously expressed, with α_2, β_2, γ_2 having the greatest AMP dependence for activation, while γ_3 is expressed more abundantly then γ_2[3]. Through overexpression of AMPKα_2, the complex of α_2, β_2 and γ_3 was believed to dominate in the muscle cell, and solely by overexpression or after activation by AICAR, lead to an increased AMPK activation as compared to endogenous complexes[13,26,3,4].

AMPK kinase (AMPKK) and AMPK can also be activated by 5-aminoimidazole-4-carboxamide-riboside (AICAR)[13]. AICAR has been reported being taken up by the cells and phosphorylated to form an AMP analogue, termed ZMP, which then activated AMPKK and AMPK similarly to AMP[15]. This chemical was therefore used as a control for the AMPKα2-overexpression experiments. The mRNA from control cells and AMPK-infected cells, either untreated or AICAR-treated, was isolated, cDNA produced and used for filter hybridization of the 67 candidate genes.

Introduction

Diabetes

Non-insulin-dependent (type II) diabetes mellitus (NIDDM) is a world-wide health problem anticipated to reach epidemic proportions over the next 10 to 20 years. The prevalence has reached 6 % in western societies and is in the range of approximately 12 % in 60 to 70 year-olds in Germany. Type II diabetes accounts for over 90 % of all diabetes and is estimated to produce health costs of 1000 Euro per patient annually. The estimated number of affected individuals for 2010 is over 215 million (WHO data from 1994). The major costs of diabetes relate to its so-called long-term complications (e.g. premature macrovascular disease, retinopathy, neuropathy etc.) that arise from poor metabolic control[5].

Glucose homoeostasis depends upon the balance between glucose production by liver and kidney and glucose utilization by insulin-dependent tissues, such as adipose tissue and muscle[6]. The utilization is highly regulated by hormones, above all by insulin and glucagon[6]. Insulin is secreted in pancreatic beta cells and glucagon in pancreatic alpha-cells[6]. Insulin suppresses the endogenous glucose production by glucagon and initiates the glucose uptake into insulin-dependent tissue[6].

In normal cells, insulin promotes the glucose uptake into the cell by docking to its membrane-receptor and triggering a signal-cascade[7]. While in insulin-dependent (type I) diabetes mellitus (IDDM) the pancreatic beta-cells are destroyed due to an auto-immune reaction, thus insulin ceases being produced, in NIDDM the beta-cells are not affected, thus insulin is still produced[7]. However, the cells develop insensitivity against insulin, known as insulin-resistance[7]. In early stages of the disease, increased insulin production compensates for insensitivity[7]. When insulin production fails to compensate for increased cellular insensitivity, the cells loose their ability to utilize glucose completely, resulting in cell-malfunctions and in a dangerous rise in blood sugar levels (hyperglycemia)[7]. In addition, insulin suppression of gluconeogenesis in the liver fails and diabetes manifests[7].

The molecular causes are not completely understood[5]. However, due to the constantly decreasing average in age of diabetes type II patients, it has been suggested that the cause cannot be merely genetically, but that also life-style plays a key role in the onset of the disease[5]. Besides genetic also environmental factors, such as diet, degree of obesity and physical activity seem to lead to the progression from normal glucose tolerance to diabetes type II[5].

AMP-activated Protein Kinase (AMPK)

Physical exercise turned out to be a key element in the control of a protein whose functions were first described in 1973[8]. But it was not before 16 years later that the different functions were related to one single protein that was named AMP activated protein kinase (AMPK)[9]. This name was given because AMPK was identified to be allosterically activated by 5'-AMP, besides the need of being phosphorylated by an upstream kinase, called AMPK kinase (AMPKK)[10,11] (**Fig. 1**). In the next years it was realized that the kinase was a kind of a gauge, monitoring levels of energy, namely the ratio of AMP to ATP, in the mammalian cells[8,12,13]. It was also shown that AMPK regulates other enzymes that are elements in different biochemical pathways, involved in utilization and/or storage of energy, e.g. fatty acid synthesis[14] fatty acid oxidation[15,16] and glycogen synthesis in liver[17].

Most importantly, AMPK was shown to promote glucose uptake into muscle-cells in an insulin independent manner[15]. It was shown that AMPK is activated by nutritional stress situations, electrical stimulated muscle contraction and physical exercise, when the AMP levels rise in relation to the ATP levels[10,18,19,20,21].

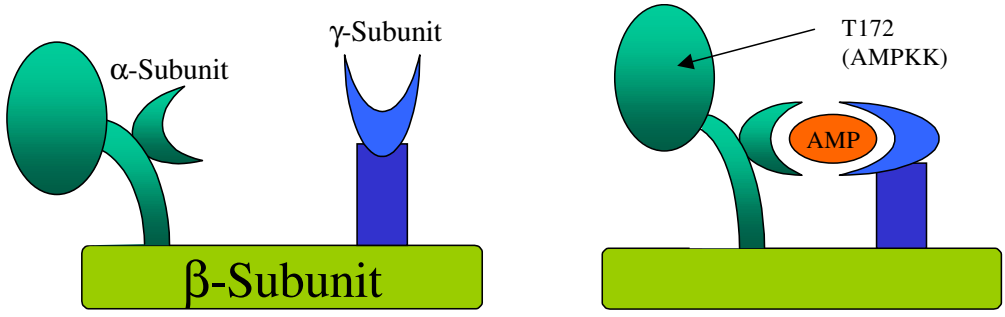


FIGURE 1: MODEL OF ACTIVATION OF AMP-ACTIVATED KINASE[12].
The left picture shows the absence while the right picture shows the presence of 5'-AMP. The α- and the γ-subunits participate in the allosterical activation by AMP. Both the catalytical domain and the phosphorylation site (threonine 172 (T172)) are located on the α-subunit. These sites are phosphorylated by AMPKK.

Soon it became clear that AMPK plays a key role in many other aspects of glucose utilization, not just by phosphorylation of downstream enzymes of different diabolic-pathways, but even by phosphorylating transcription factors[17,22,12,23,24] or insulin receptor substrate (IRS-1), a component of insulin signaling[25] (**Fig. 2**). Recently it has even been

described that AMPK plays a crucial role in obesity development and diabetes type II onset due to the importance of this protein in the fatty acid synthesis and the utilization of glucose[11].

AMPK is a protein consisting of three subunits designated α, β and γ (**Fig. 1**). The approximate masses are 63 and 38 KDa for the α- and β-subunits, respectively[8]. The γ-subunit molecular mass varies considerably among isoforms[10]. The α-subunit is the catalytic subunit containing the kinase domain, which transfers a phosphate from ATP to the target protein[8]. The β- and γ-subunits are considered regulatory components[8,10,26].

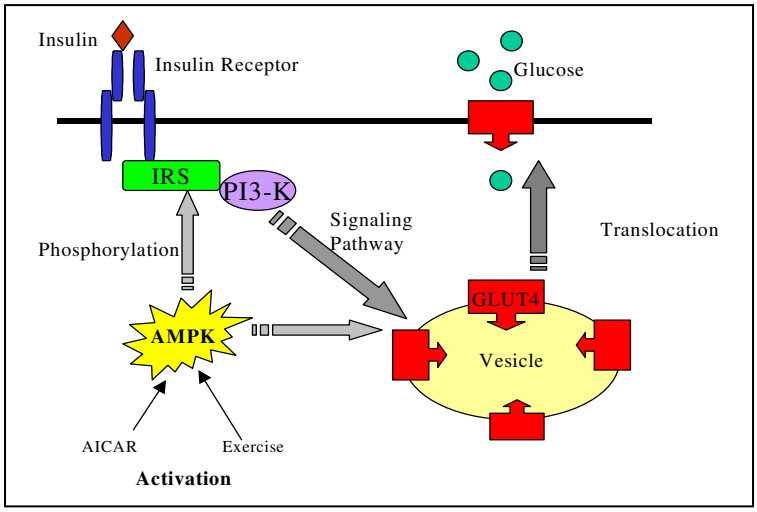
All three subunits are required for full activity[8,12]. Each subunit has two or three isoforms, designated α_1, α_2, β_1, β_2, γ_1, γ_2 and γ_3[10,11]. Information is available on tissue distribution based on immunoprecipitation studies using antibodies to all three subunits and RNA analysis[10]. α_1- and α_2-isoforms are found in skeletal muscle, but the α_2-isoform is more abundant, accounting for 80% of the total AMPK activity[18,10]. The α_2-isoform has been shown to be contraction-stimulated and is also necessary and probably sufficient to explain gene-regulatory effects on different genes[22].

Examples for regulation due to AMPK action are:

- Glucose transporter 4 (GLUT 4) translocation (see **figure 2**) was enhanced in AICAR stimulated rat skeletal muscle[27] and in AICAR treated mice[28]
- phosphoenolpyruvate carboxykinase (PEPCK) expression was repressed in AICAR stimulated H4IIE cells[23]
- Insulin receptor substrate 1 (IRS-1) was phosphorylated after AICAR stimulation in mouse C2C12 myotubes[25]
- p38 mitogen-activated protein kinase (p38MAPK) was activated due to phosphorylation, after AICAR stimulation in Clone 9 cells[29]
- acetyl-CoA carboxylase β (ACC-β) activity was decreased due to phosphorylation, after electrical induced contraction in rat muscle[18]
- fatty acid synthase (FAS) activity was reduced in rat hepatocytes by a phosphorylation/dephosphorylation mechanism, after AICAR stimulation[16]
- GLUT 1 mediated glucose transport was enhanced after AICAR stimulation of Clone 9 cells[30]
- sn-glycerol-3-phosphate acyltransferase (GPAT) was inhibited by phosphorylation after AICAR stimulation of mouse C2C12 myoblasts[24].

Facilitators of glucose transport (GLUT)

As described before, insulin triggers a signaling cascade by binding to its membrane-bound receptor. The signaling cascade diverts in the very beginning and leads to different signals[7]. The final signal for glucose-uptake into the cell is the translocation of a transporter-protein, designated glucose transporter (GLUT) 4[31]. Intracellular vesicles that translocate carry GLUT4 molecules to the membrane where the transporter becomes incorporated into the cell membrane[7]. Translocation of GLUT4 to the plasma membrane is most important for glucose uptake into the cell[32].



Explanation to figure 2:
In the basal state, GLUT4 is primarily localized within intracellular pools. The binding of insulin to the receptor, or IRS phosphorylation by AMPK initiates intracellular-signaling events that stimulate the translocation of GLUT4 to the cell surface. When GLUT4 vesicles reach the cell surface, it will dock and fuse with the plasma membrane, thus allowing the glucose-binding site on the GLUT4 molecule to become exposed to extracelullar space. This process allows GLUT4 to facilitate the movemnent of glucose into the cell.

FIGURE 2: GLUT4 TRAFFIC IN THE CELL[7].

It was shown that AMPK also leads to a translocation of GLUT4 but in an insulin independent manner[19]. This raised the question if the two different pathways that lead to similar outcomes are linked, and how AMPK triggers GLUT4 translocation (**Fig. 2**).

Adenovirus

Adenoviruses are nonenveloped, regular icosahedrons of 65 - 80 nm diameter (see **figures 3a**[33] **and 3b**[34]). The genome of adenoviruses is a linear double-stranded DNA molecule, 30 - 38 Kbp[33]. During the lytic cycle, there is an ordered expression of viral genes[33]. Adenoviruses codes for 20 - 30 polypeptides, 15 of the virus polypeptides are structural proteins[33]. Progeny virions are assembled in the nuclei of permissive cells[33]. Adenoviruses

cause upper respiratory, intestinal, and eye infections in humans[33]. Some strains of adenovirus induce tumours in newborn rodents while infections of nonpermissive cells can result in cell transformation[33].

Although adenovirus can infect a wide range of cell types, some cell types are not susceptible to adenovirus infection[35]. In part this is because of the absence of the expression of the cellular receptor for the adenoviral fiber protein (CAR)[35]. Adenovirus has structures like knobs at the tips of antenna-like fibers that are essential for adsorption to the CAR-receptor[36] (**figure 3**). Therefore the lack of CAR or a structural variant that does not allow adenovirus to dock on to the receptor prevents infection[36].

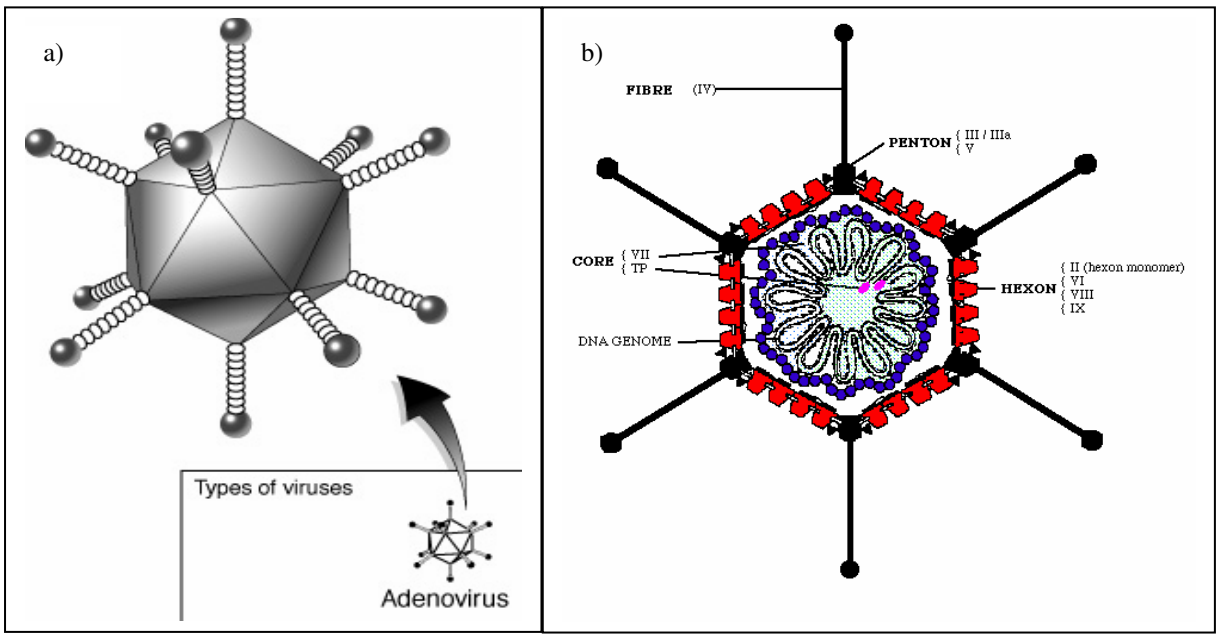


FIGURE 3: A) ADENOVIRUS STRUCTURE, B) MORPHOLOGY OF ADENOVIRUS.

Adenoviruses have been used extensively as a model system in studies of mammalian cell DNA replication, transcription and RNA processing[37] and also for overexpression studies, e.g for expression studies with overexpressed AMPKα_2 subunits[1]. The 36,000 bp double stranded DNA genome of adenovirus type 5 (Ad5) does not undergo rearrangements at high rate and the viral particle is relatively stable, making it suitable for usage as high level expression vector[37]. In nondividing cells like differentiated primary human myocytes, the viral genome may persist as an episome and continue to express for long periods of time[37].

A simplified system to generate recombinant adenoviruses is the AdEasy™ system[2] (**Fig.4**). The plasmid used is called Ad5dE13 and has a size of 33414 bp. The advantages of this system are the short production time, the possibility to monitor successful transfection

and infection due to a green fluorescent protein (GFP) insert in the vector and the fact that the recombinant viruses are incapable of proliferation in any other cell then HEK293 or 911 cell lines[1,2].

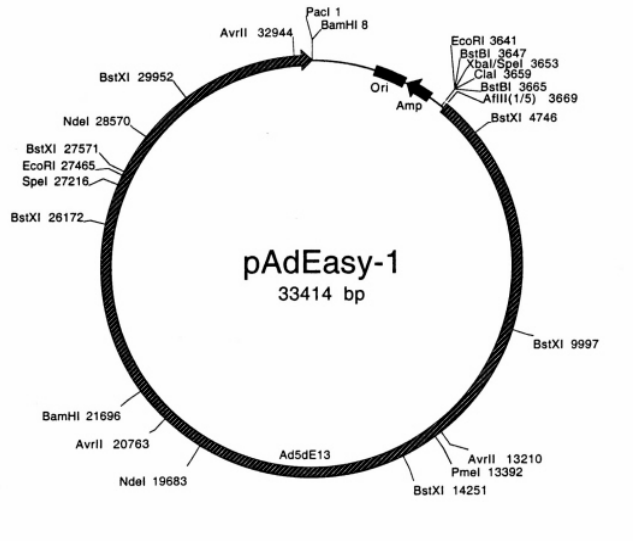


FIGURE 4: ADEASY™-SYSTEM PLASMID.

The AdEasy system provides a plasmid, named pAdEasy-1 containing the viral backbone, expressing parts of the viral proteins. Other compulsory proteins for virion production are coded in HEK293 cells (termed E1 region).

This incapability of recombinant adenovirus to replicate is due to the lack of an important DNA region, coding for several early gene products (designated "E1"), essential for replication and production of viral particles in target cells[2,3]. In the AdEasy vector the E1 region is replaced by transgenic sequences, which allows an insert of up to 10 kb[2]. HEK293 and 911 cells propagate viruses because they have been transformed to constitutively express E1 proteins, allowing to produce 10,000 plaque forming units (PFU = number of virions) per infected or transfected cell.[37].

Besides the expression of these early (E1) genes adenovirus regulates a transcriptional program that progresses with time[36]. Adenovirus infection also leads to shut-off of the transcription of many cellular genes[36]. But, selected host functions can also be activated[36].

DNA filter arrays

There are different names for DNA filter arrays, like microarrays, DNA/RNA chips, BioChips or GeneChips[38]. The array can be defined as a collection of spots of defined amount of specific species of a nucleic acid[38]. It provides the potential of monitoring expression of hundreds or even thousands of genes in parallel[39].

There are increasing numbers of commercially available DNA array formats, including glass-slide or (nylon) filter material based arrays[40]. The reasons to consider producing in-house DNA filter arrays despite the complexity of the process are various, for example the possibility to use a defined set of genes for specific questions[40]. Also cost considerations play a role, since commercially available filters are expensive in comparison to the cost of "in-house" production[40].

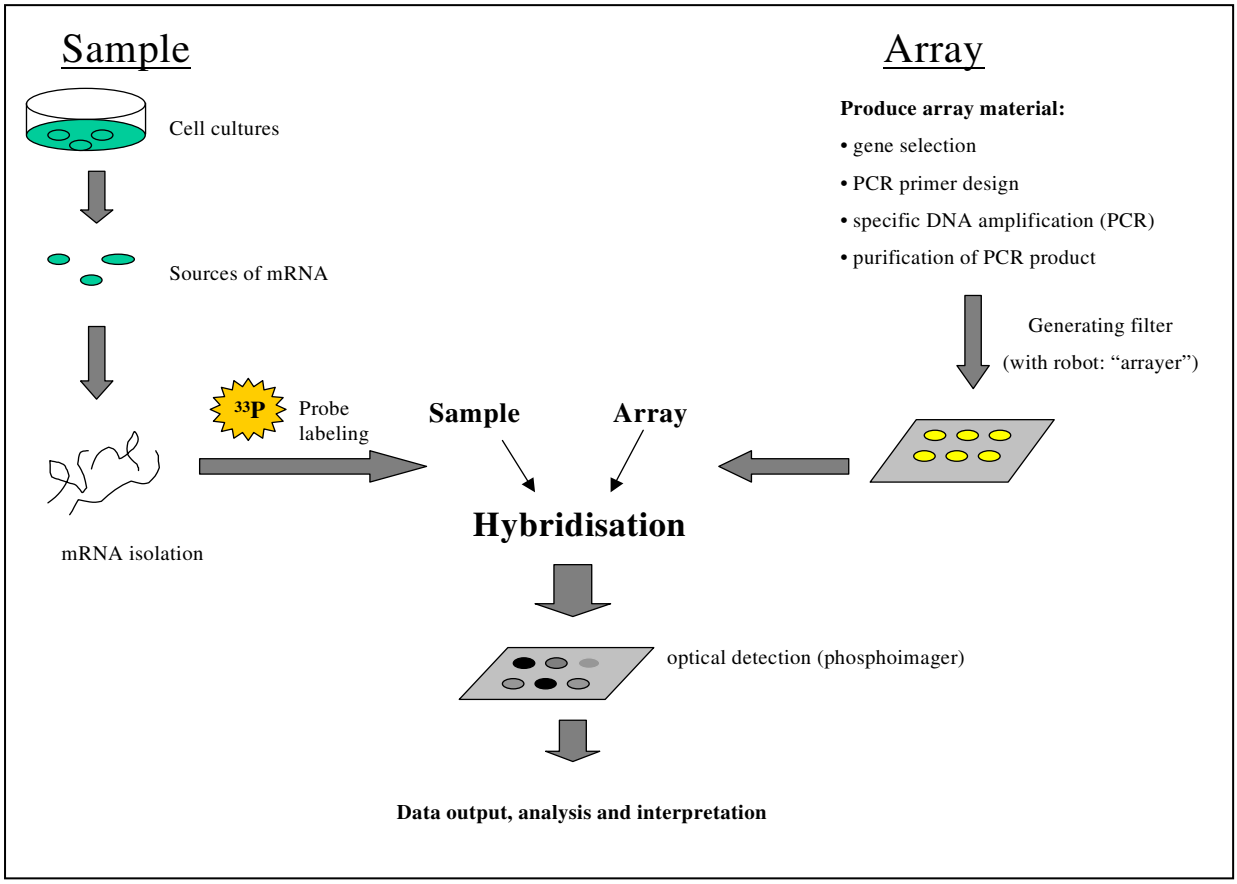


FIGURE 5: SCHEME OF EXPRESSION STUDY WITH DNA FILTER ARRAY

In order to manufacture DNA filter arrays the genes that are intended to be part of the research have to be selected[39]. The sequences are chosen from databases[38], e.g. GeneBank[41] and UniGene[42]. For DNA arrays sample DNAs are amplified by polymerase chain reaction (PCR) and the DNA molecules immobilized by high-speed robots on a solid surface such as membranes, glass or silicon chips[38]. The deposited DNA is split single-stranded by heat or alkali and cross-linked to the matrix by ultraviolet irradiation[38]. In the procedure of gene expression analysis, DNA arrays are exposed to a labeled sample of cDNA from mRNA, hybridized, and complementary sequences are detected[38](see **figure 5)**.

Aim of this study

The aims of this exam work can be divided into two parts: the *technical aspect* and a *research* oriented side of these experiments.

Technical aspects:

1. Producing a *functioning filter array* for analysis of regulated genes as the most important objective of this exam work
2. Infection, extraction and *purification of mRNA* from primary human muscle cells
3. To achieve *evidence for infection and for expression of the AMPKα_2 gene*. Firstly, by seeing an overexpression by comparing AMPKα_2 hybridization pattern between infected and uninfected (basal) muscle cells, and secondly by finding GFP expression on the filter array in infected cells

Research oriented aspects:

- To find novel gene regulation patterns due to AMPKα_2 overexpression

Materials

Cells

HEK293-cells were purchased from Microbix Biosystems, Toronto, Canada

Anna Krook and Lubna Khalili from the Karoliska Institute (Department of physiology and pharmacology) in Stockholm kindly provided human primary myocytes

Chemicals and Buffers

If not stated differently in the text, chemicals were purchased from E. Merck, Darmstadt, Germany, or Aldrich Chemie GmbH, Steinheim, Germany

PacI-restriction-enzyme from Boehringer Mannheim, Germany

Lipofectamin, OptiMemI (serum-free medium), Penicillin/Streptomycin and DMEM-medium were purchased from Gibco BRL, Life Technologies Ltd. Paisley, Scotland

Chemicals for Coulter Z2 Counter were purchased from Beckman Coulter AB, Bromma, Sweden

PBS (Phosphat-Buffered-Saline):
140 mM NaCl, 27 mM KCl, 7.2 mM $Na_2HPO_4xH_2O$, 14.7 mM KH_2PO_4, adjusted to pH 6.8-7.0 (1 M NaOH), then autoclaved

TRIS-Buffer:
1 M TRIS·HCl, adjusted to pH 7.2-8.0 (1 M HCl), then autoclaved

TE-Buffer:
10 mM TRIS·HCl (pH 8.0), 0.1 mM Na_2EDTA, autoclaved

Primers were ordered from Sigma Genosys Pampisford, Cambridgeshire, U.K.

PCR reagents from Roche Diagnostics GmbH and Clontech (Advantage PCR Kit), Palo Alto, USA

cDNA, produced at Biovitrum

Human skeletal muscle poly A+ RNA (Cat # 6541-1) from Clontech (Palo Alto, USA),

Image clones from UK HGMP, Hinxton, England

Denaturation buffer for filter-array production:
0.5 M NaOH/ 1.5 M NaCl-buffer

Neutralisation buffer for filter-array production:
0.5 M TRIS/ 1 M NaCl buffer with pH 7.5

Washing-buffers for filter-array hybridization (SSC and SDS from Clontech (Palo Alto, USA):

Buffer 1: 2x SSC, 0.05% SDS
Buffer 2: 0.1x SSC, 0.1% SDS

Composition of 20x SSC:
0.25 M Sodiumcitrate pH 7.2, 3 M NaCl, 20 mM EDTA

[α-^{33}P]dCTP from Amersham Pharmacia Biotech (AH9905), Sweden

Hybridization buffers from Clontech (Palo Alto, USA),

Sonicated salmon sperm, (CAT # 201190), Stratagene, Cedar Creek, USA

Hardware, kits and bioinformatic tools

Accession numbers for genes were found in SRS databases (Lion Bioscience Ltd, Hinxton, UK)

Gene comparisons were performed using Vector-NTI 6 from InforMax Inc., USA

Probe specificity was confirmed using BLAST homology search database, provided on the Biovitrum intranet

Primers were designed using Oligo6 design program from Molecular Biology Insights Inc., USA

4% Agarose gel from Invitrogen, Leek, the Netherlands

PCR product purification for further PCR, with Costar Centrifuge tube (Spin X, Cat.No. 8160), from Corning Costar Corporation, Cambridge, USA

PCR product purification for filter-array production was performed with a Multiscreen Filtartion System (no. MAFBN0BI0), from Multiscreen, Mosheim, France

Spectrophtomoter cuvettes from Pharmacia Biotech, Sweden

Nylon-filter-material (Hybond-N+), from Amersham Pharmacia Biotech, Sweden

Dynal mRNA extraction kit (No. 610.05) and Dynabeads (no. 610.12) from Dynal A.S., Oslo, Norway

mRNA was radioactive labeled with HotScribe first-strand cDNA labeling kit (RPN5651) from Amersham Pharmacia Biotech, Sweden

DNA was labeled using Strip-EZ DNA probe synthesis and removal kit (#1470), from Ambion, Austin, USA

Purification of labeled cDNA was performed with ProbeQuant G-50 Micro Columns (#27-5335-01), from Amersham Pharmacia Biotech, Sweden

Mineral Oil for CsCl-purification from PerkinElmer, Norwalk, USA

Dialysis of adenovirus stocks was performed with Slide-A-Lyzer Dialysis Cassette 10K (10.000 MWCO) from Pierce Chemical Company, Rockford, USA

TOPO TA Cloning kit from Invitrogen, Paisley, UK

QIAprep Miniprep from Qiagen GmbH, Hilden, Germany

PEI-Cellulose F from Merck KGaA, Darmstadt, Germany

Nylon-net for filter array hybridisation from AH Diagnostics AB, Sweden

Hybridisation container from Hybaid Ltd., Middlesex, UK

Machines

Ultrospec 3000 Spectrophotometer from Pharmacia Biotech, Sweden

Coulter Z2 particle count and size analyzer from, Beckman Coulter AB, Bromma, Sweden

Leica DM IRB microscope with EBQ100 UV-lamp from Leica GmbH, Germany

Eppendorf Centrifuge 5804R and 5417C from Eppendorf-Nethaler-Hinz GmbH, Germany

CO_2 Water Jacketed Incubator, Modell 3111 from Forma Scientific Inc., Marjetta (Ohio), USA

Beckman Ultracentrifuge Type SW41, from Beckman USA

GeneAmp 9700 (PCR), from PerkinElmer, Norwalk, USA

Agilent 2100 Bioanalyzer (with DNA500 and RNA6000nano protocols), from Agilent Technologies, Waldbronn, Germany

Speedvac System AES 2010, Savant Instruments, Holbrook, USA

Flexys-robot from Genomic Solutions Ltd., Cambridgeshire, UK

UV Stratalinker 2400, from Stratagene Cloning Systems, La Jolla, USA

Screen plates and scanner from Molecular Dynamics, Sunnyvale, CA, USA

Hybridisation oven from Hybaid Ltd., Middlesex, UK

Methods

Virus Production

Transfection

A plasmid for virus-production, containing the AMPKα2 gene, was manufactured at Biovitrum (report D. Krämer[1]). The plasmid was linearised with PacI-restriction-enzyme from Boehringer Mannheim following the manufacturer instructions and ethanol-precipitated. For ethanol-precipitation 3 µl of 3 M-sodium-acetate (pH 5) and 75 µL 99%-ethanol at –20 °C were added to 30 µl of restriction reaction mixture. After 15 min of centrifugation at 4 °C the sample was air-dried and resuspended in 20 µL deionised and sterilized water. The DNA-content was measured, using a Ultrospec 3000 Spectrophotometer from Pharmacia Biotech, Sweden. 4 µg of linearised DNA were mixed with 20 µL Lipofectamin (from GIBCO BRL) and 500 µL OptiMemI (serum-free) medium (from Gibco BRL).

HEK293 cells were cultivated in T-25-flasks containing 10% fetal bovine serum, 1% Penicillin/Streptomycin and 100x L-glutamine with 95% air and 5% CO_2 atmosphere to 90% confluence. Just before transfection the medium was removed, the cells were washed with 4 ml serum-free OptiMemI-medium (from Gibco BRL), 2.5 mL OptiMemI-medium were added and after 10 min the DNA-Lipofectamin-mix was added. After 4 hours incubation at 37 °C, the medium was removed and 6 mL fresh OptiMemI-medium were added. After one day, the efficiency of the transfection was detected by observing green-fluorescent cells, using a Leica DM IRB microscope with EBQ100 UV-lamp (from Leica GmbH, Germany) connected to a printer. After 3 days, when 1/3 to ½ of the cells were still adherent, the cells were scraped off the dishes surface, the medium transferred to 50 ml centrifugation-tubes and centrifuged at 5000 rpm for 10 min, using an Eppendorf Centrifuge 5804R from Eppendorf-Nethaler-Hinz GmbH, Germany.

The supernatant was removed and the cell-debris resuspended in 2 mL sterile PBS. The PBS-suspension was frozen using a dry-ice-methanol-mixture, thawed again in a water-bath at 37 °C and mixed thoroughly. The freeze/thaw-cycle was repeated 4 times before centrifugation at 5000 rpm for 10 min. 1 mL aliquots of the supernatant were then stored at –20 °C and later used for amplification.

Amplification

For amplification of the virus, HEK293 cells were cultivated in DMEM containing 10% fetal bovine serum, 1% Penicillin/Streptomycin and 100x L-glutamine in 95% air and 5% CO_2 atmosphere and grown at 37°C to 90% confluence in 530 cm^2 dishes. The cells were then infected with the adenoviruses from the first amplification step. After 2 days, green-fluorescent cells were observed. The cells were cultivated for one week in order to obtain a level of infection of ~90%.

Two separate sets of amplification were performed. A crude-extract of each set was obtained by scraping off the cells from the dish-surface, removing the medium and freezing 5 mL aliquots of the cell-containing medium at –20 °C. The cells were lysed by this freezing step. Before using the extract for infection the suspension was centrifuged at 5000 rpm for 10 min and the supernatant removed for infection. Using the supernatant an end-point-titration was performed.

End-point-titration

HEK293 cells cultivation conditions were as follows: DMEM containing 10% fetal bovine serum, 1% Penicillin/Streptomycin and 100x L-glutamine with 95% air and 5% CO_2 atmosphere at 37°C. A 1 mL portion of HEK293 cells was thawed and grown until circa $2x10^6$ cells were obtained, to be transferred to a 96 well plate. The cells were counted, using a Coulter Z2 particle counter and size analyzer, from Beckman Coulter AB, Bromma, Sweden, following the manufacturer's instructions. The amount of cells in 200 µL DMEM medium transferred into each well was approximately $2x10^4$ to obtain 90 % confluence after one day of incubation at 37°C. Dilutions of the crude-extract were made in DMEM and 20 µL used per well, i.e. undiluted, 1:10, 1:100 etc. up to $1:10^8$. The medium in the 96 wells was substituted by the virus dilutions. After 15 min of incubation at 37°C with 95% air and 5% CO_2 atmosphere, 180 µL DMEM were added per well.

After 48 hours of incubation, the amount of GFP-cells was determined in the wells that contained between 70 and 170 infected cells. The amount of PFU (PFU-plaque forming units) per milliliter was calculated: number of counted cells divided by the dilution factor (gives cells in 20 µL of the dilution), multiplied with 50 gives infected cells per milliliter. On basis of the PFU the amount of virus to be used in later experiments was determined.

Purification of virus for stocks

1/20 IGEPAL (Octylphenoxy)polyethoxyethanol) CA-630 was added to the crude extract. The mixture was shaken for
20 min at room temperature. Successful lysis was controlled using a Leica DM IRB microscope. The suspension was then transferred into centrifugation bottles and centrifuged at room temperature for 15 min at 20,000 x g (Eppendorf Centrifuge 5804R). The supernatant was transferred into a new bottle. 1/20 of a sterile 2,5 M NaCl in 20 % PEG-2000-solution was added and the mixture was shaken over night. The solution was centrifuged for 15 min at 20,000 x g, the supernatant removed and the pellet – containing the virus – resuspended in a minimal amount of PBS. Centrifugation of the suspension for 10 min at 7,000 x g and 4°C (Eppendorf Centrifuge 5804R) separated the cell-debris as pellet from viruses in solution.

4.4 g of CsCl were dissolved in 8 mL of the virus solution. After covering with mineral oil, the solution was centrifuged at 32000 rpm at 10°C for 16 to 27 h in a Beckman Ultracentrifuge Type SW41. The virus band was recovered from the tube using an auto-pipette. The suspension was adjusted to a final concentration of 10 mM Tris, 4% sucrose and 2 mM $MgCl_2$ at pH 8.0. Aliquots were kept at -80°C.

Dialysis of CsCl-stocks

In order to use a virus stock for infection on cells again, the stock solution must be dialysed. The dialysis exchanges the toxic CsCl in the stock solution for NaCl from PBS. The dialysis was carried out with a Slide-A-Lyzer Dialysis Cassette 10K (10.000 MWCO) from Pierce Chemical Company, Rockford, USA. Following the manufacturer's protocol, the cassette was used 2 times for 30 min in PBS. The cassette's loading volume was approximately 500 µL, the volume of used PBS was circa 500 mL each time. A test infection on HEK293 cells in a 96 well plate was performed- as described in the " End-point-titration"-part of this chapter- in order to determine if toxicity was abolished.

Production of DNA-Filter-Arrays

Gene-specific DNA sequences

A list of 83 proteins (including positive/negative controls) was assembled in accordance to the requirements described in the "Introduction" chapter. By using the names of the proteins, their SRS accession numbers were determined and the human cDNA sequences fetched in FASTA format from public databases. SRS™ is a data retrieval system that integrates heterogeneous databanks in molecular biology and genome analysis (Lion Bioscience Ltd, Hinxton, UK).

In order to produce probes specific for the coding regions (CDS) of the genes, the positions of the CDS were determined using SRS databases and then extracted from the full sequences obtained in the first step. Families of genes were then compared to allow choice of primers specific for each family member. Bioinformatic tools were used to process the data, i.e. Vector-NTI 6 from InforMax Inc. The design of primers for PCR was carried out using the obtained data in Oligo6 design program from Molecular Biology Insights Inc. (USA). The primers were designed to be specific for the gene and to give a product of 140 to 160 bp. In some cases, i.e. in the case of gene families, the primer sequences and/or the product sequences were compared in BLAST database to exclude similarities with other genes or members of the gene-family. The primers were ordered at Sigma Genosys (Pampisford, Cambridgeshire, U.K).

Design of gene-specific oligo-nucleotides and production of gene specific DNA fragments

PCR reactions were performed using reagents from Roche Diagnostics GmbH or Clontech (Advantage PCR Kit) following the manufacturer's general instructions for PCR's. The purchased primer were diluted and added to the reaction mixture, as suggested by the producers manual..

Using SRS and Biovitrum databases, it was determined which templates could be used to execute PCR with. The different templates were either cDNA, produced at Biovitrum, (from: human genomic-, liver-, brain-, fetal brain-, heart- and testicle-RNA), Image clones (from UK HGMP, Hinxton, England) or Biovitrums plasmids.

The reaction products of these PCR's were isolated by a gel electrophoresis on a 4% Agarose gel (Invitrogen), purification by cutting out the product gel fragment and spinning it in a Costar Centrifuge tube (Spin X, Cat.No. 8160) at 14000 rpm for 5 min.

These purified products were then used to optimize the PCR. Two optimized PCRs were then performed for all genes in order to obtain a large amount of product for manufacturing the filter-arrays. The PCRs of 50 µL for each product were pooled (total volume 100 µl) and purified, to exclude pollution, e.g. with primer fragments, using a Multiscreen filtration system (no. MAFBN0BI0, from Multiscreen, Mosheim, France) with 96 well filter plates, following the provided protocol. The final elution volume was approximately 40 µL of TE Buffer. Concentration of DNA was measured using an Agilent 2100 Bioanalyzer following the DNA500 protocol.

Production of gene-filter-arrays

In order to obtain equal concentrations of DNA products, specific amounts of solution, containing the purified DNA-fragments with known concentration, measured using an Agilent 2100 Bioanalyzer following the DNA500 protocol, were transferred into a 96-well plate and the solvent evaporated in a Speedvac System AES 2010 (Savant Instruments, Holbrook, USA). The DNA was dissolved in equal amounts of deionised and sterilized water. From this concentrated solution dilutions of 1:10 and 1:100 were made, and used for gridding on the nylon-membrane (Hybond-N+, from Amersham Pharmacia Biotech). The 3 different concentrations were 10 ng/µL, 1 ng/µL and 0.1 ng/µL. The concentration was measured for some samples using the AgilentDNA500 protocol. Duplicates of approximately 0.6 µL were gridded to the filter. A flexys-robot from Genomic Solutions Ltd., Cambridgeshire, UK, was used for the gridding, following the manufacturer's instructions.

The DNA on the nylon filter material was denaturised in 0.5 M NaOH/ 1.5 M NaCl for 10 min and neutralized in 0.5 M TRIS/ 1 M NaCl (pH 7.5) for 5 min. In order to covalently bind DNA to the filters, UV light was used (UV Stratalinker 2400, from Stratagene), using energy of 120 mJ on both filter-sides.

Cloning of PCR products

To be able to quickly produce large amounts of the gene fragments, e.g. in order to confirm the correctness of the used filter array fragments (by e.g. DNA sequencing), and for other future experiments, the products were cloned into commercially available vectors. A TOPO TA Cloning kit (from Invitrogen, Paisley, UK) was used, following the manufacturer's protocol. The amount of fresh PCR product used for cloning was approximately 2 μL. A part of the obtained bacteria suspension was used to perform a plasmid DNA isolation, following the protocol of the QIAprep Miniprep (from Qiagen GmbH, Hilden, Germany). The other part of the bacteria suspension was used to produce a glycerol stock, with a concentration of 20% of glycerol and stored at -80°C.

Infection of primary human myocytes

Titration of produced virus in primary human myocytes

Anna Krook and Lubna Khalili from the Karoliska Institute (physiology and pharmacology) in Stockholm kindly provided primary human myocytes. Lubna Khalili has performed the isolation, cultivation and differentiation of the cells. She provided 96 well plates with a cell density of approximately 90% of differentiated primary human muscle cells. The cells had been differentiated for two days to obtain a maximum of infection efficiency, as it had been determined in earlier experiments performed at the Karolinska Institute.

The cells originated from biopsies of rectus abdomius muscle from different subjects and have an internal registration number at Karolinska Institute. For the titration of the virus extracts in human myocytes cells of subject HS1 III were used, for time course experiments HS1 IV and for the three main infections it were the subjects HS1 IV, HS13 and HS13 respectively.

The first extraction of mRNA from these cells was used for experiments to improve labeling and hybridization (see discussion). The second set of mRNA was used for the main labeling experiment, while the third was stored at -80°C for further experiments in the future.

A dilution series of the crude virus extracts of the AMPKα2 construct (Titer: $4x10^8$ pfu/mL) and a GFP-control construct (Titer: $7.9x10^7$ pfu/mL) were made and infections carried out, using the provided 96 well plate (**Table 1**). When amplifying the virus two

separate sets of infection and crude extraction were performed, therefore both sets were tested separately (AMPK1, AMPK2).

DMEM with 2% FBS was used for dilution. 100 µL of each dilution step were used per well. After 1 h of incubation at 37°C, 100 µL DMEM were added. After incubation o/n at 37°C, the medium was replaced by fresh DMEM containing 2% FBS. The cell's level of fluorescence was observed using a Leica DM IRB microscope with EBQ-100 UV-lamp from Leica GmbH, Germany.

Table 1: Scheme of dilution for titration in primary human myocytes (96 well format)

		1	2	3	4	5	6
AMPK ampl. 1	A	no virus	30+20	40+10	1*10E-01	1*10E-02	1*10E-03
	B	no virus	30+20	40+10	1*10E-01	1*10E-02	1*10E-03
	C						
AMPK ampl. 2	D	no virus	30+20	40+10	1*10E-01	1*10E-02	1*10E-03
	E	no virus	30+20	40+10	1*10E-01	1*10E-02	1*10E-03
	F						
Control (GFP)	G	no virus	no virus	no virus	2.5*10E-2	2.5*10E-3	2.5*10E-4
	H	no virus	no virus	no virus	2.5*10E-2	2.5*10E-3	2.5*10E-4

		7	8	9	10	11	12
AMPK ampl. 1	A	1*10E-04	1*10E-05	1*10E-06	1*10E-07	1*10E-08	undiluted
	B	1*10E-04	1*10E-05	1*10E-06	1*10E-07	1*10E-08	undiluted
	C						
AMPK ampl. 2	D	1*10E-04	1*10E-05	1*10E-06	1*10E-07	1*10E-08	undiluted
	E	1*10E-04	1*10E-05	1*10E-06	1*10E-07	1*10E-08	undiluted
	F						
Control (GFP)	G	2.5*10E-5	2.5*10E-6	2.5*10E-7	2.5*10E-8	2.5*10E-9	2.5*10E-10
	H	2.5*10E-5	2.5*10E-6	2.5*10E-7	2.5*10E-8	2.5*10E-9	2.5*10E-10

Fluorescent (i.e. infected) and dead cells were counted, respectively estimated. Highest infection efficiency with lowest levels of cell death were determined comparing these results. The required dilution for the most efficient infection to be used in cultivation dishes of other size was estimated on the basis of this data.

Infection for time course experiments

To determine what the most efficient infection and cultivation duration would be, in order to obtain the highest yield of mRNA extract, a time course experiment was performed. 100 mm petri dishes with primary human myotubes of a cell-density of circa 90% were infected with the AMPKα_2 construct (Titer: $4x10^8$ pfu/mL) and a GFP-control construct

(Titer: $7.9x10^7$ pfu/mL) viruses. 3 mL dilution were used per dish. The dilution for the AMPKα_2 construct was 1:10 and for the GFP construct 1:40 in DMEM with 2% fetal bovine serum. The cells were incubated for 2 h at 37°C, then 3 mL DMEM with 2% fetal bovine serum were added. Medium was exchanged after 24 h.

The cells were then harvested with Dynal lysis buffer (from Dynal A.S., Oslo, Norway) after 30 h, 48 h and 72 h (**Table 2**), and used for mRNA extraction, following the manufacturers mRNA extraction kit (No. 610.05) protocol, using 20 µL of elution buffer. The mRNA concentration was then measured using a Ultrospec 3000 Spectrophotometer from Pharmacia Biotech, Sweden, Germany). The probes were diluted 1:10 and measured, following the instructions provided with the Spectrophotometer, using 10 µL cuvettes from Pharmacia Biotech, Sweden.

For calculation of the total amount of mRNA extract, the results were multiplied with the dilution factor (10 x) and the total volume of elution buffer (~20 µL). The mRNA was stored at -80°C until being used for labeling and hybridization.

Table 2: Number of plates used for time course experiment infection

virus	30 hours RNA	48 h RNA	72 h RNA
No infection	1 plate	1 plates	1 plate
AMPK	1 plate	2 plates	1 plate

Main experiment for mRNA extraction

The main experiment of infection, for harvest of mRNA was carried out three times (**table 3**). Set one was used for labeling and hybridization experiments in order to improve the efficiency of techniques used. The second set was used for hybridization. The third was stored at -80°C for future use.

100 mm petri dishes with primary human myotubes of a cell-density of circa 90% were infected with the AMPKα_2 construct (Titer: $4x10^8$ pfu/mL of crude extract) and a GFP-control construct (Titer: $7.9x10^7$ pfu/mL of purified virus) viruses. 3 mL dilution were used per dish. The dilution for AMPKα2 construct was 1:10 and 1:40 for the GFP construct in DMEM with 2% fetal bovine serum. The cells were incubated for 2 h at 37°C, then 3 mL DMEM with 2% fetal bovine serum were added. Medium was changed after 24 h. After 72 h

incubation one plate per experiment was used for AICAR stimulation. The cells were stimulated using 1 mM AICAR for 20 min.

Table 3: Numbers of plates used for the main infection experiment

Virus + treatment	RNA 1st experim.	RNA 2nd experim.	RNA 3rd experim.
No infection	2 plates	3 plates	2 plates
No inf. + AICAR	2 plates	3 plates	2 plates
GFP	2 plates	3 plates	2 plates
GFP + AICAR	2 plates	3 plates	2 plates
AMPK	4 plates	3 plates	2 plates
AMPK + AICAR	4 plates	3 plates	2 plates

The cells were harvested with Dynal lysis buffer (from Dynal A.S., Oslo, Norway), and used for mRNA extraction, following the manufacturers mRNA extraction kit (No. 610.05) protocol. The mRNA concentration was measured using an Agilent 2100 Bioanalyzer (with RNA6000nano protocol, from Agilent Technologies, Waldbronn, Germany). The mRNA was kept at -80°C until being used for labeling and hybridization.

Labeling, hybridization and screening

Purification of mRNA

The cells that were harvested with 1 mL Dynal's lysis buffer (from Dynal A.S., Oslo, No. 610.05) per 100 mm plate (see chapter "infection of primary human muscle cells") were used for extraction of mRNA. The "mRNA direct kit" with Dynabeads (no. 610.12) was used in accordance to the manufacturer's protocol. The final elution was in 20 µL 10 mM Tris-HCl. mRNA concentration and pollution with rRNA was measured using an Agilent 2100 Bioanalyzer, following the manufacturer's RNA6000nano protocol.

PCR products as template for ^{33}P-labeling of cDNA

In order to test specific genes on the filter array, selected DNA probes from the PCR reaction were labeled using ^{33}P-ATP. The Strip-EZ DNA probe synthesis and removal kit (from Ambion, Austin, USA) was used, following the manufacturer's instructions. The

amount of used template DNA was ~35 ng. The labeling efficiency and reactions purity was estimated by performing a thin layer chromatography (TLC) as described in the labeling kit instructions, using PEI-Cellulose F from Merck KGaA, Darmstadt, Germany. The TLC was then exposed to screen plates for 15 min and scanned, using phosphoimager screens and scanner from Molecular Dynamics, Sunnyvale, CA, USA, following the manufacturer's instructions. The successfully labeled probes were then used for hybridization to the DNA filter arrays.

Test ^{33}P-labeling of cDNA by reverse transcription of purchased mRNA

In order to test the labeling efficiency and to test the filter arrays furthermore, purchased human skeletal muscle poly A+ mRNA (from Clontech, Palo Alto, USA), was labeled with HotScribe first-strand cDNA labeling kit (RPN5651) from Amersham Pharmacia Biotech, Sweden, using ^{33}P-dCTP. The labeling was performed in accordance to the manufacturer's protocol. In addition to the described procedure in the manual, 5 µL EDTA (0.5 M EDTA) were added, so that the resulting concentration was 10 mM EDTA. This prevents degradation of cDNA as described in the "troubleshooting" section of the protocol. The total amount of used mRNA was approximately 0.5 µg to 1 µg, labeled with [α-^{33}P]dCTP from Amersham Pharmacia Biotech (AH9905), Sweden. The labeling efficiency and purity of reaction product was estimated by performing a thin layer chromatography (TLC) as described in the labeling kit instructions, using PEI-Cellulose F from Merck KGaA, Darmstadt, Germany. The TLC was then exposed to screen plates for 15 min and scanned, using screen plates and scanner from Molecular Dynamics, Sunnyvale, CA, USA, following the manufacturer's instructions. The successfully labeled probes were then used for hybridization to the DNA filter arrays.

^{33}P-labeling of cDNA by reverse transcription of extracted mRNA

The purified mRNA was ethanol precipitated: pellet paint, 0.5 volumes of 7.5 M NH_4OAc and 2.5 volumes of absolute ethanol (stored at –20°C) were added to the mRNA sample and mixed vigorously. Immediately the mixture was centrifuged at 12,000 x g in a

5417C microcentrifuge from Eppendorf-Nethaler-Hinz GmbH, Germany, at room temperature for 20 min.

The supernatant was removed, the pellet washed with 0.5 mL of 80% ethanol (stored at -20°C) and centrifuged at 12,000 x g at room temperature for 5 min. The ethanol was removed carefully and the washing repeated for one additional time. The pellet was air dried and resuspended, using 12 µL of RNase free water. The resuspended mRNA was labeled using the HotScribe first-strand cDNA labeling kit (RPN5651) from Amersham Pharmacia Biotech, Sweden. The labeling was performed in accordance to the manufacturer's protocol. In addition to the described procedure in the manual, 5 µL EDTA (0.5 M EDTA) were added, so that the resulting concentration was 10 mM EDTA. This prevents degradation of cDNA as described in the "troubleshooting" section of the protocol. The total amount of used mRNA was approximately 0.5 µg to 1 µg, labeled with [α-^{33}P]dCTP from Amersham Pharmacia Biotech (AH9905), Sweden.

The labeling efficiency and purity of reaction products was estimated by performing a thin layer chromatography (TLC) as described in the labeling kit instructions, using PEI-Cellulose F from Merck KGaA, Darmstadt, Germany. The TLC was then exposed to screen plates for 15 min and scanned, using screen plates and scanner from Molecular Dynamics, Sunnyvale, CA, USA, following the manufacturer's instructions. The successfully labeled probes were then purified and used for hybridization to the DNA filter arrays.

Purification of labeled cDNA

The labeled probes were purified using ProbeQuant G-50 Micro Columns, (from Amersham Pharmacia Biotech, Sweden), following the manufacturers protocol. The purification efficiency and reactions purity was estimated by performing thin layer chromatography (TLC) as described in the labeling kit instructions, using PEI-Cellulose F from Merck KGaA, Darmstadt, Germany.

The TLC was then exposed to screen plates for 15 min and scanned, using screen plates and scanner from Molecular Dynamics, Sunnyvale, CA, USA, following the manufacturer's instructions. The successfully labeled probes were then used for hybridization to the DNA filter arrays.

Hybridization to filter-arrays

In the following, mentioned buffers and solutions were from Clontech (Palo Alto, USA), and the method developed on the basis of Clontech's protocol for hybridization.

The filters were put on a nylon-net (from AH Diagnostics AB, Sweden) and transferred into hybridization container from Hybaid Ltd., Middlesex, UK. 1.5 mg of sheared salmon testes (from Stratagene, USA. Cat # 201190) DNA was heated for 5 min at 95-100°C, quickly chilled on ice and added to 15 mL ExpressHyb solution, pre-warmed at 50°C. 10 ml of this mix were added to the hybridization container and pre-hybridized for 30 min at 60°C in a hybridization oven (from Hybaid Ltd., Middlesex, UK). The ^{33}P-labelled probe was mixed with 1.5 µg of sheared salmon testis DNA, heated for 5 min at 95-100°C and added to the remaining ExpressHyb solution.

The pre-hybridization mix was substituted for the labeled hybridization mix and hybridized o/n at 60°C. The hybridization mix was poured out and the filters were washed four times for 30 min at 60°C with washing buffer1 (Buffer 1: 2x SSC, 0.05% SDS), then two times for 20 min at 60°C with washing buffer 2 (Buffer 2: 0.1x SSC, 0.1% SDS). The filters were drained, but not dried and immediately transferred into a plastic bag that was heat-sealed.

The sealed filter-arrays were exposed to phosphoimager screens o/n and then screened in a scanner (screens and scanner from Molecular Dynamics, Sunnyvale, CA, USA) following the manufacturers manual.

Results and discussion

Virus production

Production and storage

The plasmid construct to express virions in HEK293 cells contained a GFP gene. This gene, when expressed in a cell, codes for a protein that produces a green fluorescent illumination, when observed under UV light. Thus, when the cells were transfected with the virus plasmid, a green fluorescence could be determined using UV light already after 24 h, increasing to a maximum intensity after 48 h. **Figure 6** shows an example of the cells appearance in an uninfected state, and after infection.

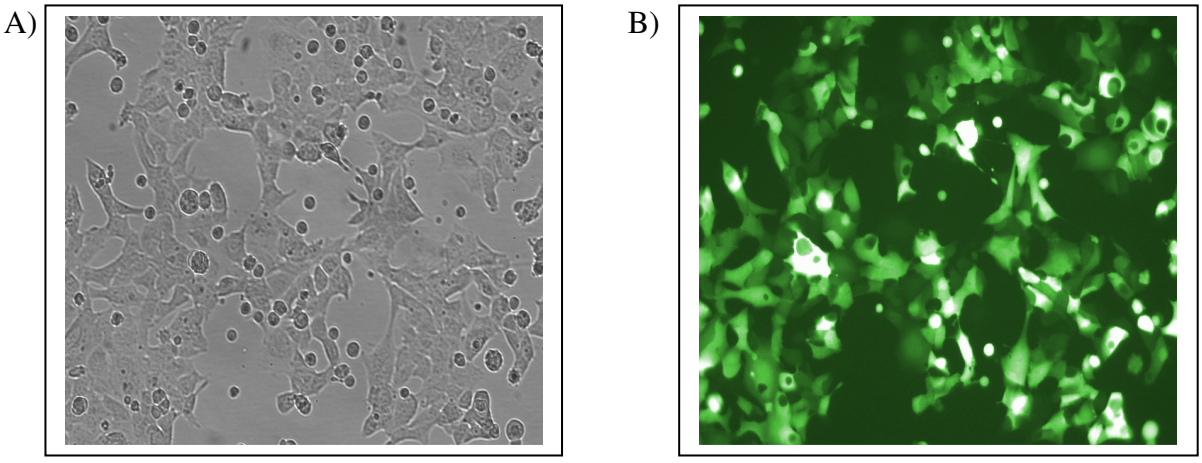


FIGURE 6: HEK293 CELLS UNDER DAYLIGHT (A) AND UV LIGHT (B), AFTER ADENOVIRUS INFECTION.

A crucial observation was made, concerning the storage of extracted virus from virus production with HEK293 cells. After the lysis of virus-containing cells in PBS (see "methods"), due to repeated freeze and thaw cycles, the suspension was stored at -20°C for some weeks (i.e. first virus extraction after transfection). A major decrease in titer could be observed (in relation to the transfection titer), when an amplification step was performed using this extract. Only very few infection spots, i.e. green cells were observed. The decrease in titer was most probably the result of insufficient storing conditions. In case of long term storage, i.e. for more than half a year, a storage buffer has to be used, as described in the chapter "virus production: purification of virus for stocks". For storage between 6 to 12 months a crude extract is sufficient, as described in the chapter "virus production: amplification". This crude extract was stored at -20°C for several weeks, while performing

the different infections ("time dependence experiment" and "main experiment for mRNA extraction"), without any observable drop in titer.

When performing the large scale virus amplification in HEK293 cells, the virus extract in PBS was used, resulting in the low level of infection (as described above). In order to get the desired high level of infection, the cells were grown for about a week, with a change of medium every second day (also see "methods" section). The few infected cells proliferated, producing the virus until they lysed, infecting the surrounding cells in great numbers. After 7 days 90 % of the cells were infected (**Figure 6**) and a crude extract obtained, as described in the "methods" section. This crude extract was used for the determination of the PFU.

Determination of the value of PFU

To determine the PFU of the crude extract of AMPKα_2-virus as described earlier ("methods"), the infected cells in 20 µL of the virus suspension with a dilution of $1x10^{-5}$ were counted three times per well for both construct sets. The mean was calculated and used for PFU calculation (**table 4**). When the number of infected cells is divided by the dilution factor the result gives PFU per 20 µL (the volume of crude extract taken, when performing the infection for the titration experiment). When multiplied with 50 the result is plaque forming units per milliliter.

The calculation of PFU was carried out as follows:

$$\frac{\text{infected cells (PFU)}}{\text{Dilution factor}} \text{ X } 50 \rightarrow \left(\frac{\text{PFU}}{20\ \mu\text{L x } 50} \right) = \left(\frac{\text{PFU}}{\text{mL}} \right)$$

The value of PFU per milliliter gives an estimate of the volume of crude extract to use for infection. If possible cells to infect in an experiment are counted prior to infection. When dividing the PFU value by the number of counted cells the result gives an estimate of how many infectious particles per cell are used, when applying one milliliter of virus suspension. This is termed "multiple of infection" (MOI). We considered that a MOI close to one is desired, i.e. one infectious particle (virion) per cell. Therefore the volume used was chosen in a way, so that approximately one virion per cell was used for infection.

Table 4 shows the numbers of infected HEK293 cells after 2 days of infection, when the GFP activity was at its maximum (**Fig. 7,** also see "discussion"). Two separate infections were performed, in order to get higher yields of virions and for security reasons, i.e. in case one set would have been infected with bacteria or mycoplasms. The counts were performed 3 times per well and per dilution in order to minimize counting errors.

Table 4: Number of infected HEK293 after 48 h.

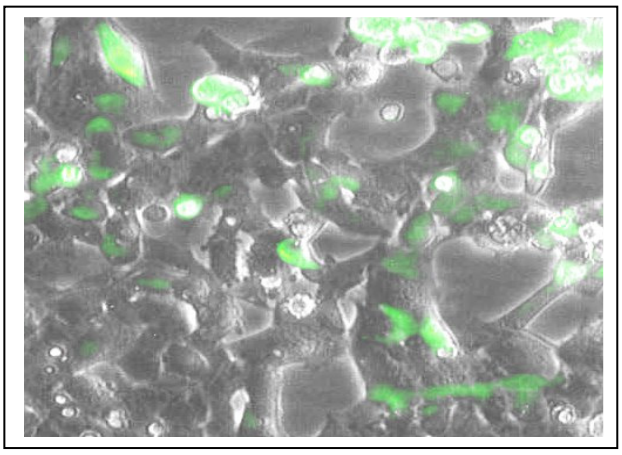
Counts	1	2	3	Sum	Mean	PFU
Constr. 1	111	87	70	268	89	4.47E+08
Constr. 2	98	169	146	413	138	6.88E+08

As can be seen, the values vary considerably. This is mainly based on difficulties to distinguish single infected cells from infected cell clusters. The dilution used for calculation, as shown in **table 4,** was $1x10^{-5}$ of the crude extract. This dilution gave an approximate number of 100 infected cells per well. Less than 100 infected cells per well lower the precision of the result (because of statistical effects, due to random variation in infection efficiency), while more than 100 infected cells per well become too difficult to count. Firstly because clusters of infected cells become more frequent and secondly, because it becomes very time consuming to count them.

FIGURE 7: INFECTED HEK293 CELLS

Performing an end-point-titration, HEK293 cells were infected using a crude adenovirus extract. The image shows the infected cells under UV-light, 48 h after infection. Merging a daylight- and a UV-light capture and digitally enhancing the green color obtained the picture.

Clusters of infected cells are hard to distinguish from single infected cells. To minimize counting errors the counting was repeated three times per well and dilution step.



The cells shown in **figure 7** are captured after 48 h. As described further later in this chapter, it turned out that an infection time of 24 h is more sufficient for an end-point titration. The reason is that there is a possibility of cells producing large amounts of virus, lysing and re-infecting their neighboring cells after 48 h. This risk is much lower after 24 h. Thus, a longer

cultivation time after infection could be resulting in titers appearing considerably higher than they really are. Nevertheless, the obtained figures were sufficient to estimate the amounts of virus used in the following titrations in primary human myocytes.

Infection of primary human myocytes and mRNA extraction

Titration results

This experiment was performed, in order to determine infection efficiency of adenovirus in primary human myocytes. Again by observing the green fluorescent cells the infection was monitored.

It is known that adenovirus does not infect different types of cells in the same way (see "introduction: adenovirus" for details). To enter the cell the virus uses a mechanism that starts with docking to specific cell receptors. Varying amounts of these receptors as well as differences in the receptor composition have an effect on the infectivity[36].

Another imaginable effect on the efficiency of infection can be the matrix that contains the virus. Especially when using crude extracts one could expect disturbances in cell growth due to impurities. The AMPK construct was used as a crude extract, i.e. a suspension containing medium and cell proteins besides the adenovirus, while the GFP was a purification, i.e. the pure virus in a buffered solution (see "methods").

Table 5 shows the dilution scheme, used for the titration, with the dilutions marked that provided the best results. **Table 6** presents the cell death after 48 h of infection and the infection rate.

Table 5: Scheme of dilution for titration in primary human myocytes (96 well format)

		1	2	3	4	5	6
AMPK ampl. 1	A	no virus	30+20	40+10	1*10E-01	1*10E-02	1*10E-03
	B	no virus	30+20	40+10	1*10E-01	1*10E-02	1*10E-03
	C						
AMPK ampl. 2	D	no virus	30+20	40+10	1*10E-01	1*10E-02	1*10E-03
	E	no virus	30+20	40+10	1*10E-01	1*10E-02	1*10E-03
	F						
Control (GFP)	G	no virus	no virus	no virus	2.5*10E-2	2.5*10E-3	2.5*10E-4
	H	no virus	no virus	no virus	2.5*10E-2	2.5*10E-3	2.5*10E-4

		7	8	9	10	11	12
AMPK ampl. 1	A	1*10E-04	1*10E-05	1*10E-06	1*10E-07	1*10E-08	undiluted
	B	1*10E-04	1*10E-05	1*10E-06	1*10E-07	1*10E-08	undiluted
	C						
AMPK ampl. 2	D	1*10E-04	1*10E-05	1*10E-06	1*10E-07	1*10E-08	undiluted
	E	1*10E-04	1*10E-05	1*10E-06	1*10E-07	1*10E-08	undiluted
	F						
Control (GFP)	G	2.5*10E-5	2.5*10E-6	2.5*10E-7	2.5*10E-8	2.5*10E-9	2.5*10E-10
	H	2.5*10E-5	2.5*10E-6	2.5*10E-7	2.5*10E-8	2.5*10E-9	2.5*10E-10

The dilutions that were used for the following time dependence experiment are marked in **table 5 and 6**. The dilutions used were 1:10 for the AMPKα_2-1 construct and 1:40 for the GFP control virus. With these dilutions the highest infection rates were achieved while occurring cell death was regarded acceptably low. There was no detectable infection in higher dilutions than $1x10^{-3}$ for AMPK virus and $1x10^{-4}$ for GFP virus.

Table 6: cell death and the infection rate after 48 h of infection

	[%]		1	2	3	4	5	6	7	8	9	10	11	12
AMPK1	Death	A	0	~40	~30	~20	<5	<1	<1	<1	<1	<1	<1	~40
	Infection	B	0	>90	>90	>90	~40	<5	0	0	0	0	0	>90
		C												
AMPK2	Death	D	0	~50	~30	~30	~10	<5	<1	<1	<1	<1	<1	~50
	Infection	E	0	~100	~50	~20	~10	<5	0	0	0	0	0	100
		F												
Control (GFP)	Death	G	0	0	0	~50	~50	~30	<5	<5	<5	0	0	0
	Infection	H	0	0	0	~80	~50	~40	<5	0	0	0	0	0

Even though the titers of the GFP and the AMPKα_2 constructs vary considerably, i.e. $4x10^8$ pfu/mL for AMPKα_2-1, $7x10^8$ pfu/mL for AMPKα_2-2 and $7.9x10^7$ for the GFP-control construct, infection efficiency appears to be similar in almost comparable dilutions (1:10 for AMPK and 1:40 for GFP). One would expect for example a higher dilution for AMPKα_2-1, in comparison to GFP, because of the higher titer. A reason for this not to be the case is most probably due to differences in the protocol followed when performing a titration to determine the PFU. Therefore leading to incomparable titers. The GFP virus was produced and titered by a different laboratory (Hindrik Mulder from Lund University, personal contact).

Furthermore, experiments performed by Karin Nielson (personal contact) indicated that using our protocol ("methods") a cultivation of 48 h after infection was too long. When performing an end-point titration in HEK293 cells it might be necessary to limit cultivation

time to 24 h after infection. This is because HEK293 will proliferate and produce virus and infect other cells within a 48 h time frame, thus leading to a too high titer.

Furthermore the GFP construct was purified, while the AMPKα_2 virus was a crude extract. There have been considerations that crude extracts could contain enzymes that influence virus stability, or even lead to virus degradation. Confirming experiments have not been performed, though Hindrik Mulder from the Lund University (personal contact) confirmed that his laboratory frequently performs successful experiments, based on crude extracts.

Additionally it has been considered that in case of infection levels of close to 100%, cells could have taken up more then one virion. Thus a difference in infected cells would not be distinguishable, though amounts of virions taken up per cell might vary considerably. This circumstance is described as MOI (multiple of infection)[37]. The MOI gives a value for the number of virions per cell, used for infection. We concluded that a MOI of 1 (virion/cell) appears desirable. Because the number of cells of human myocytes per petri-dish was unknown, no determination of MOI could be performed.

These considerations also might also partially explain the reason for the varying amount of dead cells. A higher MOI might lead to multiple infections per cell and increase the stress the cell has to undergo, due to high expression levels of proteins transcribed from the inserted virus plasmid[36]. Another reason might be the fact that the cells originated from different individuals, therefore not being susceptible to infection in an equal way resulting in different cell death rates (**Table 9**).

Anyhow, the results of this titration experiment allowed us to calculate the exact amounts of virus that had to be used in the following time-dependence experiments.

Time-dependence experiment

A time frame infection experiment was set up, in order to determine the best timeframe for infection efficiency in combination with low cell death rates (**Table 7**) and highest yields of mRNA extract (**Table 8**). It was expected that expression levels could have been varying because of e.g. inhibitory effects. The idea behind this was, that expression of proteins is often regulated by a feedback mechanism. That means that the expressed protein inhibits its own expression, e.g. by binding to the DNA of the protein's gene sequence and therefore repressing the transcription machinery (similar to e.g. "Lac-operon"-model[43]). In the

case of AMPKα_2 it would have also been imaginable that the protein inhibits transcription factors responsible for its transcription, since AMPK's effect on some transcription factors has been described before (see "Introduction: AMPK"). As shown in **table 8**, there was no indication of such effects. The total amount of transcribed mRNA appears to be constant over time.

Table 7: Time dependence experiment results, with amount of death cells and infectivity

	30 hours		48 h		72 h	
virus	death	infection	death	infection	death	infection
No infection	None	none	None	none	None	none
GFP	not performed		~10%	~50%	~20%	~50%
AMPK	~10%	~30%	~20%	~70%	~30%	~70%

mRNA extraction was measured via absorption (i.e. nucleic acids at 260 nm in relation to proteins at 280 nm) with a spectrophotometer (see "methods" chapter). It has to be remarked that for AMPK 36 h a failure in the purification process lead to an incorrect low yield in mRNA, therefore the numbers are not shown in **table 8**. However, repetition was not necessary, because a comparison of the measured values was sufficient to decide about the timeframe for the main experiment.

Table 8: mRNA extraction from time dependence experiments

		Conc. [□g/□L]			Total (20 □L)
Time [h]	Probe	1. Elution	2. Elution	Sum	
36	no inf.	0,062	0,030	0,092	1,84
	AMPK	-	-	-	-
48	no inf.	0,040	0,022	0,062	1,24
	AMPK	0,055	0,007	0,062	1,24
72	no inf.	0,052	0,026	0,078	1,56
	AMPK	0,066	0,028	0,094	1,88

The varying mRNA concentration most probably depends on the varying amount of cells, grown on the 100 mm petri-dishes. When performing the different infections it was observed that while the cells sometimes had grown to an almost 95 % confluence, other times there were large gaps between the cells. This was partly because the cultivation procedures for primary human myocytes made it impossible to count the cells prior to seeding, leading to different numbers of cells per plate. But, more crucial seemed the number of passages. It has

been observed that the more often the cells were passed on, the fewer cells survived the passage and the lower was their confluency.

The experiment showed that mRNA could be obtained, using the Dynabeads extraction system. Furthermore the duration of infection did not occur to have a mayor impact- if any impact at all- on the yield of mRNA, despite the seemingly increased cell-death over time. When entering the cell, adenovirus starts a transcription program that evolves over time[36]. Possibly this may lead to an increasingly demanding nutritional situation and finally to increased cell death over time. Though, one has to keep in mind that cell-death naturally occurs over time, also without infection. Thus it was decided to cultivate the cells for 72 h after infection.

Main experiment for mRNA extraction

Sets of cells were infected with the GFP virus, the AMPK construct and one set was left uninfected (see "methods"). Varying numbers of plates were used for each experiment („methods", **table 3**). Shortly before harvesting, half of the plates of each set of experiments were incubated with AICAR, a chemical AMPK activator (see "introduction: AMPK"). After 72 h of cultivation, the primary human myocytes were harvested for mRNA extraction. The mRNA was extracted separately, measured and then pooled.

The confirmation of successful extraction of mRNA was achieved by measuring mRNA concentration with the Agilent RNA6000nano protocol (**table 10**). The details of the Agilent measured concentrations can be found in the appendix (also see **Fig. 8** for gel picture).

This experiment was carried out three times. After infection a certain level of cell-death occurred and was monitored **(table 9)**. The aim was to label the mRNA with radioactivity (i.e. ^{33}P), and use it for hybridization to DNA filter arrays, in order to confirm expression of GFP and AMPKα_2 and to possibly even find AMPK regulated genes.

Table 9: Cell death after infection for mRNA extractions

Infection	1		2		3	
Subject	(HS1 IV)		(HS13)		(HS13)	
Construct	AMPK	GFP	AMPK	GFP	AMPK	GFP
Celldeath [%]	~30	~35	~10	~30	~35	~35

A certain amount of cell death occurred possibly due to cell stress when infection happens as described before (**Table 9**). This can possibly also be explained by a multiple of infection (MOI). A higher MOI leads to multiple infections per cell and increases the stress the cell has to undergo due to virions entering the cell and due to high expression levels of proteins transcribed from the inserted virus plasmid (see „introduction: adenovirus“). Additionally, Lubna Khalili (personal contact at Karolinska Institute) reported that she had observed that primary human myocytes were very sensitive in comparison to e.g. cell lines like HEK. That is in terms of their capability to survive disturbances like e.g. lack of nutrition. It is therefore imaginable that infection raises cell death in primary human myocytes.

Table 10: Amount of mRNA from human muscle cells for main experiment (2. infection).

	AMPK		AMPK+AICAR		GFP		GFP+AICAR		uninfected		uninf.+AICAR	
	1	2	1	2	1	2	1	2	1	2	1	2
mRNA [ng]	1043	538	988	412	831	650	596	523	760	708	133	671
Sum [ng]	1581		1400		1481		1119		1468		804	

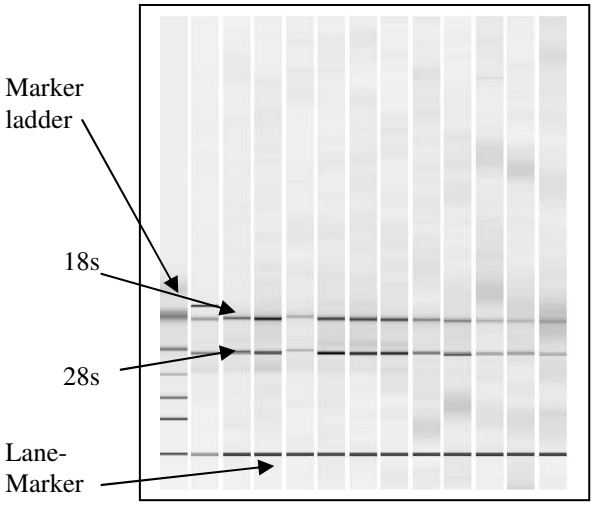


FIGURE 8: MRNA MEASUREMENT WITH AGILENT PROTOCOL.

The figure shows a gel picture from an Agilent6000nano mRNA measurement. Agilent computer software performs a translation of measured data into a gel-like picture.
The mRNA is visible as stretched out smear, because of its varying sizes. The ribosomal RNA though is very distinct, because it is clearly represented by its two distinct bands, consisting of the 18s and 28s bands.

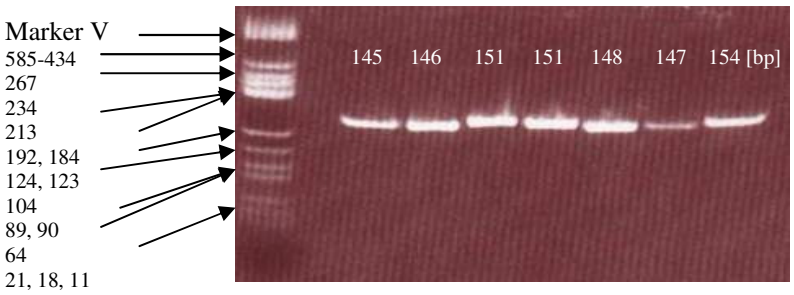
Purity of the mRNA extractions and quality in terms of size distribution of the fragments could be estimated using the Agilent gel prints (**figure 8**). The sharper the 18s and 28s bands, the better the quality in terms of purity. A successful purification results also in an almost equal distribution of mRNA fragments over the whole spectrum, i.e. all over the gel. In case of degraded mRNA one will find empty spaces on the gel or clusters of higher concentrated small mRNAs. Quality, concentration and total amount of mRNA was sufficient to be used in the following labeling and hybridization experiments.

PCR products for filter array production

The principle of DNA filter arrays is that a labeled DNA probe, e.g. radioactive or fluorescent, binds to an anti-sense DNA anchored, i.e. covalently bound, on the filter surface. Gene specific primers were designed and purchased. Using those primers, gene specific products of a length of 140 to 160 bp were produced in PCR reactions. The products were controlled performing gelelectrophoresis on 4 % agarose gels, containing ethidiumbromide. The size was subject to control (size marker used for comparison). Also the purity- i.e. to obtain only one product per reaction- of the PCR reactions was important. The single bands of the gene products of correct size were excised and extracted from the gel (UV-light capture in **figure 9**, also see methods).

FIGURE 9: AGAROSE GEL OF PCR PRODUCTS

The picture (UV-light capture) shows products with a size of 140-160 bp (lanes 3-9). The first lane shows a marker V, from Boehringer Mannheim (Germany)



In case there were impurities, i.e. more then one product or no specific product, the reactions were improved until purity was satisfactory. In few cases (e.g. PEPCK gene, data not shown) PCR amplifications resulted in multiple bands or smearing, even after several attempts to improve the reaction conditions[40]. In those cases the product was extracted directly from the gel and used for application on the filter array. Sometimes yields of products were low (weak bands on the gel). Then the reactions were improved (annealing temperature etc.) in order to get higher yields. The PCR products were then amplified in three to four single reactions (see "methods").

After high scale amplification, the concentrations of DNA products were measured spectrophotometrically (Agilent). The products were then purified, i.e. cleaned from primer and in some cases from side products and measured again. A sample of a typical gel, as obtained using an AgilentDNA500 protocol, is shown in **figure 10** (For detailed data see appendix).

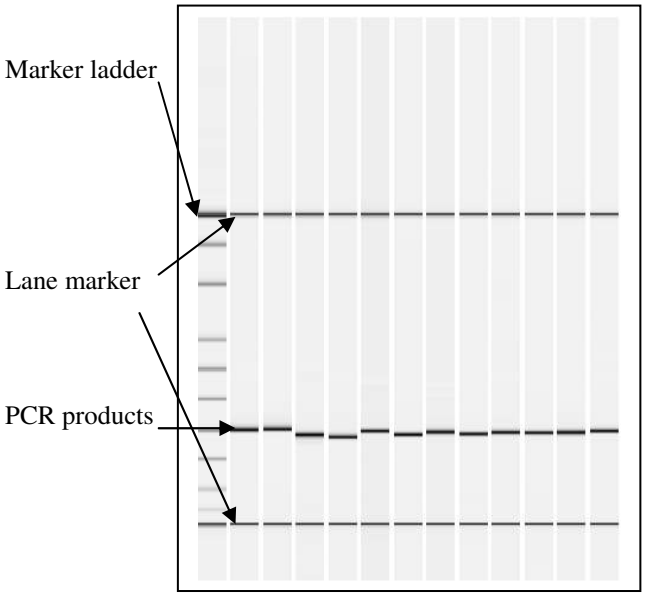


FIGURE 10: AGILENT DNA500PROTOCOL-GEL OF PCR PRODUCTS

The image shows an example of an Agilent "gel" of PCR products after purification, measured with the AgilentDNA500 protocol. The Agilent software translates the measured data into a gel-like picture. The DNA concentration and fragment size data is shown in the appendix.
The first left lane shows the agilent specific marker, called ladder, which is used to automatically determine the fragment sizes.

The obtained DNA concentrations were the basis for the calculation of the amount of DNA that had to be covalently bound to the filter array. The filter arrays were produced as described in the ”methods” chapter and used for the following hybridization experiment with radioactive labeled cDNA. **Figure 11** shows the gridding scheme of the genes put on the filters.

Filter array gridding

The genes E1 to C2 are a selection of the subunits of the AMPK protein (**figure 11**). AMPKα_2 (gene F1) was used to control successful overexpression of the subunit. The other subunits deliver a possibility to determine if AMPKα_2 has an effect on their expression.

Genes A10 to D11 are control genes, i.e. genes that allow comparison of signal intensity of different expression levels and unspecific hybridization. For example A10 has a high, B10 medium and C10 low expression levels in most cells. D10 to G10 are sensors for unspecific hybridization. A11 is a sequence of the green fluorescent protein (GFP) gene and can therefore be used to in confirm infection with the recombinant adenovirus constructs that express the GFP. B11 to C11 are human, yeast and *E.coli* genomic DNA. Finally, the rest of the genes are potential candidates of being regulated by AMPK. Some of them already have been demonstrated being regulated in earlier published studies (see “introduction: AMPK”). Note that for confidential reasons some of the names were changed.

	1	2	3	4	5	6	7	8	9	10	11
A	Acetyl CoA carb.a		creatine kinase B	GLUT4	GSK3, alpha	L-pyruvate kinase	PEPCK cytosolic	PKB gamma	gene A9	beta-actin	GFP
B	ACC2	AMPK gamma 2	creatine kinase M	gene B4	GSK3, beta	MEF2-A	Phosphoinositol dep. kin. I	gene B8	Spot14	gamma-actin	genomic DNA human
C		AMPK gamma 3	gene C3	gene C4	Hexokinase II (muscle)	MEF2-B	PI3K alpha 85	gene C8	STAT5B	PP1	genomic DNA yeast
D		Calmodulin kinase I	gene D3	glycogen synthase (liver)	Hexokinase IV (liver)	MEF2-C	PI3P-5K	gene D8		Phospholipase A2	genomic DNA *E.coli*
E	AMPK alpha 1	Calmodulin kinase IV	Erk2	glycogen synthase (muscle)	HSL	gene E6	PKA alpha	PP2c	UCP1	Highly basic protein	
F	AMPK alpha 2	CBP	Erk5	GRB 14	Insulin receptor	gene F7		PP5	UCP2	Ribosomal protein S9	
G	AMPK beta1	CPT1 (liver)	FAS	GRB 2	IRS1			PTP1B	UCP3	G3PDH eller GAPDH	
H	AMPK beta2	CPT1 (muscle)	GLUT2	GRB10	IRS2	p38 MAPK beta	PKB alpha	gene H8	gene H9		

FIGURE 11: DNA FILTER ARRAY APPLICATION SCHEME
(grey squares represent empty filter array areas)

Confirmation of DNA filter array function via hybridization experiments

Improvement of labeling efficiency

In accordance to the labeling kit manufacturer the usage of fresh isotope and the purity of mRNA extracts determined labeling efficiency. Experiments performed at Biovitrum, by Kerstin Larsson (personal communication) had shown that higher purity increased the amount of incorporated isotope. The amount of rRNA pollution of the extract was a factor that lowered labeling efficiency. Therefore, additionally to the purification of mRNA, an ethanol precipitation was performed.

Labeling purchased human muscle mRNA

In order not to waste any of the extracted mRNA from primary human myocytes, and to control labeling efficiency, a test labeling was performed using purchase human muscle mRNA. The labeling was carried out, as described in the "methods" section, using ^{33}P. The labeling was purified and applied on a thin-layer chromatography (TLC) (**figure 12**). The

TLC was heat sealed in a plastic bag. Phosphoimager-screens were exposed to the TLC for circa 15 min.

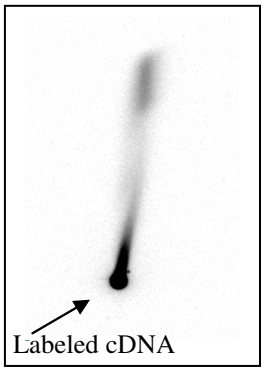


FIGURE 12: TLC OF LABELED PURCHASED MRNA.

After scanning a phosphoimager screen of unpurified TLC screen often 3 fractions can be seen. The fraction closest to the application point is the properly incorporated cDNA. This fraction should be left after purification. The middle fraction is free dCTP (or other nucleotide, used for labeling), while the upper fraction represents free $^{33}PO_4^{3-}$.

Successful purifications, where almost only the labeled cDNA was obtained, are shown in **figure 12, 15 and 18**. Thus being applicable for the hybridization experiments.

Hybridisation of labeled cDNA from purchased muscle mRNA

The ^{33}P labeled mRNA (i.e. sscDNA) was used for hybridization to the produced filter arrays. After washing the hybridized filters they were sealed in plastic bags, exposed to phosphoimager screens and scanned (**figure 13**). The exposure time was ~24 h.

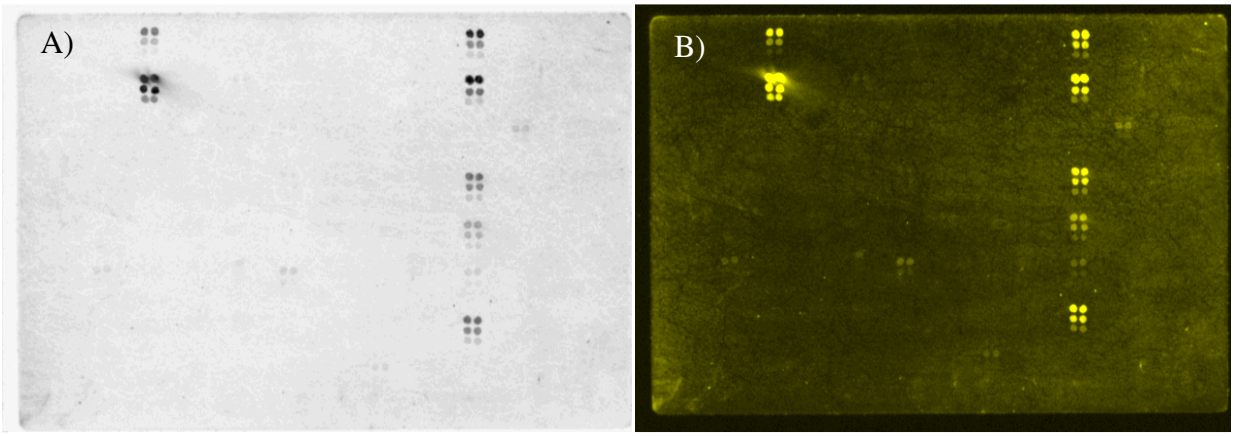


FIGURE 13: HYBRIDIZATION EXPERIMENT WITH HUMAN MRNA. (A) ORIGINAL SCAN, (B) COLOR MANIPULATION.

Filter array spots that have bound radioactive labeled mRNA appear black after scanning (**figure 13 a**). The darker the hybridization mark the more mRNA has bound to the filter array spot. Some of the hits are too weak to be easily visible on the printed picture. Anyhow, they were detected using computer software by manipulating the pictures quality

(e.g. **figure 13 b**) provided by the manufacturer of the scanner (Molecular Dynamics, USA). Therefore **figure 14** comprehends the data by indicating hybridization hits as red squares.

FIGURE 14: RESULTS OF MRNA TEST LABELING.

Empty parts of the filter are marked grey, hybridisation hits are marked red, and genes without hybridisation result are left blank. See also "results: filter array production" for more details.

	1	2	3	4	5	6	7	8	9	10	11
A	Acetyl CoA carb.a		creatine kinase B	GLUT4	GSK3, alpha	L-pyruvate kinase	PEPCK cytosolic	PKB gamma	gene A9	beta-actin	GFP
B	ACC2	AMPK gamma 2	creatine kinase M	gene B4	GSK3, beta	MEF2-A	Phosphoinositol dep. kin. I	gene B8	Spot14	gamma-actin	genomic DNA human
C		AMPK gamma 3	gene C3	gene C4	Hexokinase II (muscle)	MEF2-B	PI3K alpha 85	gene C8	STAT5B	PP1	genomic DNA yeast
D		Calmodulin kinase I	gene D3	glycogen synthase (liver)	Hexokinase IV (liver)	MEF2-C	PI3P-5K	gene D8		Phospholipase A2	genomic DNA *E.coli*
E	AMPK alpha 1	Calmodulin kinase IV	Erk2	glycogen synthase (muscle)	HSL	gene E6	PKA alpha	PP2c	UCP1	Highly basic protein	
F	AMPK alpha 2	CBP	Erk5	GRB 14	Insulin receptor	gene F7		PP5	UCP2	Ribosomal protein S9	
G	AMPK beta1	CPT1 (liver)	FAS	GRB 2	IRS1			PTP1B	UCP3	G3PDH eller GAPDH	
H	AMPK beta2	CPT1 (muscle)	GLUT2	GRB10	IRS2	p38 MAPK beta	PKB alpha	gene H8	gene H9		

The experiment provided first evidence that filter production had been successful. Anyhow, many of the genes were expected to be expressed ubiquitously in human muscle cells and therefore result in hybridization, but they gave no signal. For example AMPKα_2 was expected to always be expressed to an extend in human muscle cells that would be detectable. On the other hand it was not evaluated what the sensitivity of the filters was. Therefore the question was if there was no signal for AMPKα_2 because of expression levels below detection threshold, or if there were filter production errors. To evaluate the possibility of filter production errors, an experiment using labeled PCR products that had been used for filter manufacturing was performed.

Labeling and hybridizing selected ^{33}P labeled PCR products

In order to evaluate the possibility of filter array manufacturing errors, three genes that were expected to be expressed in human muscle cells and were regarded especially important for the following final experiment were selected. The genes selected were AMPKα_2, PEPCK and MEF2c. With AMPKα_2 indicating successful infection and overexpression of the subunit, while PEPCK and MEF2c were expected being regulated by AMPK.

The PCR products of those genes that had been produced for filter array manufacturing were labeled with ^{33}P and applied on TLC to control incorporation of ^{33}P into the cDNA (**figure 15 a**). The reactions were purified and also applied on TLC to control the purity of the labeled cDNA (**figure 15 b**).

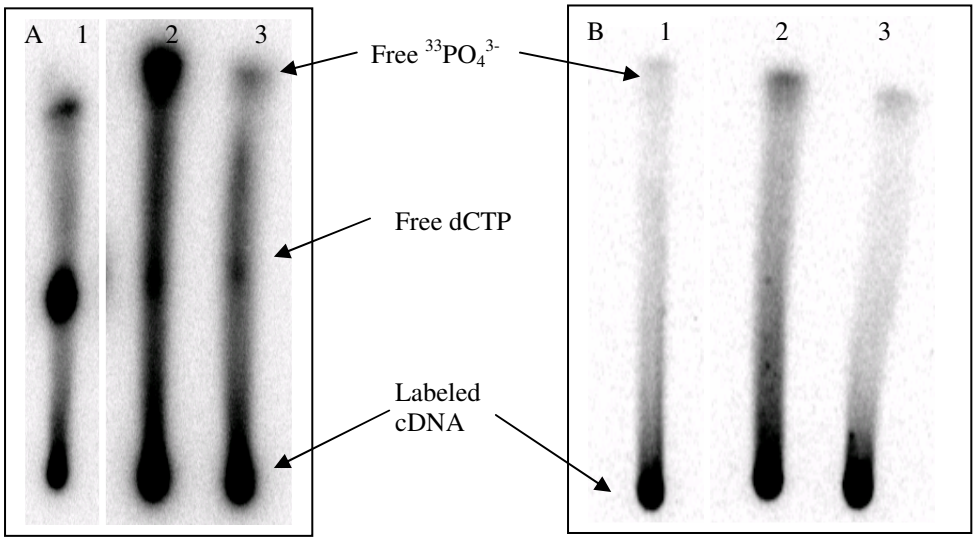


FIGURE 15: TLC OF LABELED PCR FRAGMENTS.

A) Before purification
1. AMPKα2
2. PEPCK
3. MEF2c

B) After purification
1. AMPKα2
2. PEPCK
3. MEF2c

The purified labeling mixture (**fig. 15 b**) indicated that labeling had resulted in cDNA with large amounts of incorporated ^{33}P, thus being applicable for hybridization to the DNA filter arrays. The hybridization results are shown in **figure 16**. It has to be noted that some hybridization marks were too weak to be visible on a print out, therefore hits that had been detected using a computer imaging program (provided with the scanner from Molecular Dynamics, USA) were framed in red and labeled in accordance to the gridding scheme in **figure 11**.

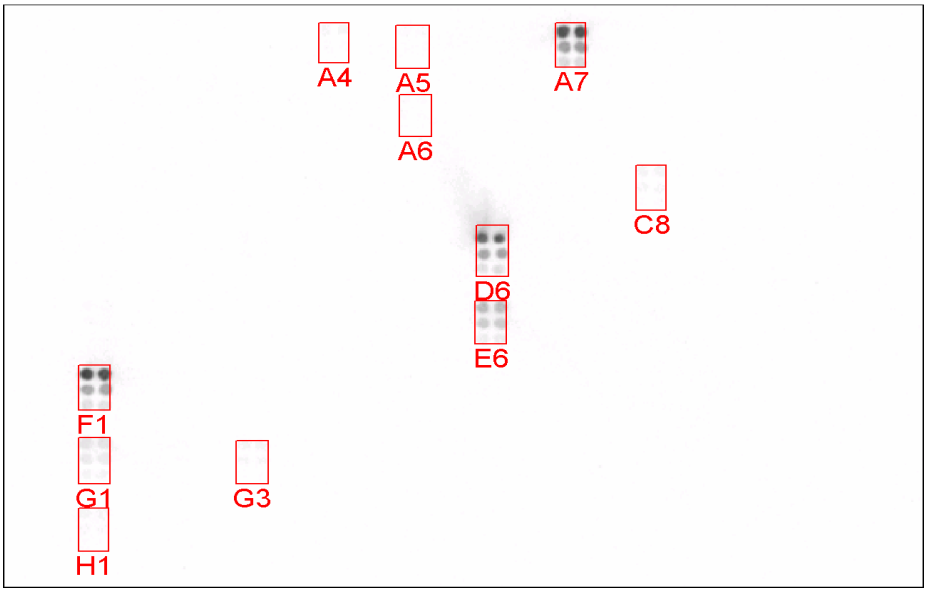


FIGURE 16: HYBRIDIZATION WITH LABELED PCR PRODUCTS.

F1	AMPKα2
G1	**AMPKβ1**
H1	**AMPKβ2**
G3	**FAS**
A4	**GLUT4**
A5	**GSK3α**
A6	**GSK3β**
D6	MEF2c
E6	**geneE6**
A7	PEPCK
C8	**geneC8**

The result indicates that unspecific hybridization occurred with the red printed genes (**figure 11**). Anyhow, the selected genes were hybridized and gave a strong signal, thus being further proof that filter production had been successful. Taken together with the results from the mRNA hybridization this indicated sufficient specificity for large cDNA fragments. Thus the weakness of some signals in the hybridization experiment with purchased human muscle mRNA must be based on other considerations.

Weak hybridization signals

It was assumed that occurring changes might go undetected in case not all cells were infected, i.e. if there were changes in too few cells to be detected. For technical reasons the infection had to be carried out at Karolinska Institute, where the infection of the myocytes could not be monitored (due to a lack of UV microscopes), when cultivating cells for the main experiment. In order to achieve the maximum of infection with at the same time lowest cell death one would have to monitor infection on a daily basis. As our experiments (performed with Lubna Khalili, Karolinska Institutet) hint, the efficiency of infection is depending on more factors than just the titer of the virus suspension. As mentioned before, the way of application of the virus, cultivation procedures and cell passages seem to have an influence. Especially when working with primary cells varying infection efficiency occurs to be a crucial factor due to their sensitivity (in accordance to Lubna Khalili's experience, as mentioned above).

In order to gain higher yields of mRNA more cells could be cultivated. The weakness of the hybridization patterns might be based on the low expression of some target genes and could possibly be compensated by usage of higher amounts of mRNA for labeling and hybridization. This circumstance seems to be indicated by this experiment that had been performed, using PCR fragments, which gave strong signals for the selected genes (for concentrations see "methods"). Also the mRNA extraction protocol might have to be improved, in order to get higher yields and lower rRNA pollution. An amount of 3-5 µg of mRNA might be suitable. Anna Krook from the Karolinska Institute (personal contact) reported that they recently have been successful to extract 3-5 µg of mRNA from similar amounts of cells, as used in our experiment, using a different protocol. In the time frame of an exam work it turned out to be too time consuming to perform those improvements.

Hybridization and cross-hybridization

The hybridization with labeled PCR fragments gave some cross hybridization. This might derive from the fact that small labeled fragments, when hybridized to small fragments (both 150 bp) are more likely to hybridize unspecifically[40]. Additionally to the increased likelihood of binding, a rather mechanistic model was considered to be applicable to explain the circumstances further. If a small fragment hybridizes with only a few base pairs, the binding energy might be sufficiently strong to resist attempts to break it by washing. Because in contrast, if the bound fragment has a long "tail" of DNA floating in the washing buffer, greater forces affect the fragment and thereby eventually breaking the bond. This might sufficiently explain the cross hybridization.

Another possibility of increased background signal and unspecific binding could be degradation of cDNA when being labeled (in accordance to the labeling kit manufacturer's manual. See "methods"). The small oligonucleotides would have a higher chance of homology to the filter array DNA. The first mentioned explanation is regarded more plausible, being confirmed by the fact that even though there are strong signals when hybridizing with cDNA from purchased human muscle mRNA, no cross hybridization was detected (i.e. no large number of weak, seemingly unspecific signals). If degradation would have occurred when using PCR products for a labeling and hybridization experiment, degradation should also occur producing cDNA from purchased human muscle mRNA, thus also produce unspecific signals, which appeared not to be the case.

Washing the hybridized filter arrays is a crucial step to abolish unspecific hybridization and avoid strong background signals, which decrease signal contrast. **Figure 13** shows a fuzzy hybridization for gene B3. A similar result can be seen in **figure 16**, for hybridization mark D6. This, as well as the mentioned unspecific hybridization in **figure 16**, indicates that washing conditions can be improved further.

The reason that hybridization to genomic yeast cDNA (**figure 14:** C11) occurred may be that yeast, in contrast to human genomic cDNA, has only few introns. This might raise probability of homology of the yeast CDS that are closely related to human CDS.

Anyhow, this experiment indicated that the DNA filter arrays appeared to be manufactured correctly, thus being usable for the final experiment, i.e. performing hybridization with labeled cDNA from primary human myocyte mRNA extracts.

Hybridization with extracted mRNA from primary human myocytes

Labeling of extracted mRNA from primary human myocytes

This final experiment was intended to give results that would allow making a statement about the infection success (GFP expression), the expression of AMPKα_2 and a possible regulatory effect of AMPK on the selected genes of the DNA filter array.

The isolations and purifications of mRNA from primary human myocytes, as described above, were used for labeling with ^{33}P. After labeling the efficiency of ^{33}P incorporation was controlled, performing a TLC (**Fig. 17**). The labeling was purified from free isotope and free dCTP. The TLC, performed to control the purification success, is shown in **figure 18**.

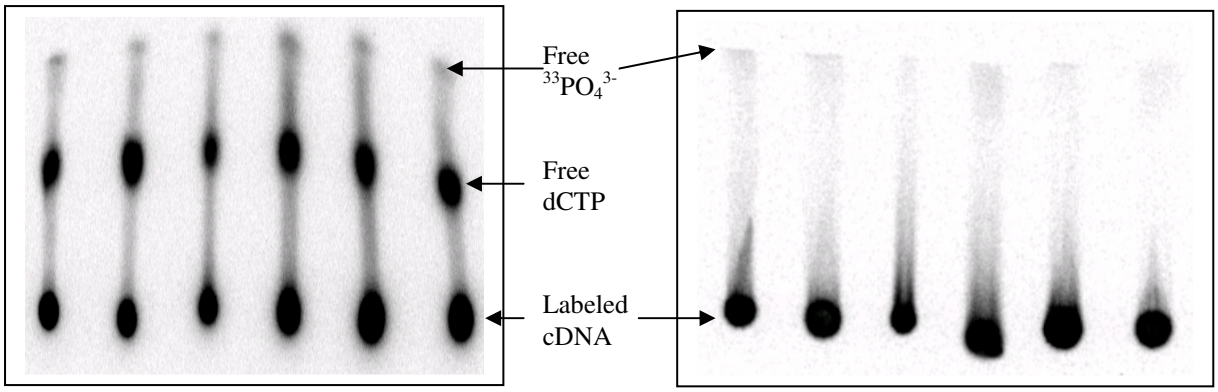


FIGURE 17: TLC OF LABELED MRNA EXTRACTION

FIGURE 18: TLC OF PURIFIED MRNA LABELING

The major fraction left over, after successful purification, was the cDNA with incorporated ^{33}P isotope. The purified labeling reaction mix was further processed for hybridization.

Filter hybridization with labled cDNA from primary human myocyte mRNA

The hybridization with the labeled mRNA from primary human myocytes was carried out as described in the "methods" chapter. The hybridized filter arrays were heat sealed in plastic bags and used for exposure to phosphoimager screening plates and scanned. The scanning results after 4 d of exposure are presented in **figure 19 to figure 24** (All figures were colored for improved contrast).

Because hybridization hits might be too weak to be visible on a print. Therefore they were marked and labeled in accordance to the gridding scheme of the filter arrays, after they had been confirmed using computer software tools (see "methods")(**figure 11**).

Uninfected primary human myocytes with and without AICAR stimulation

The results of labeled cDNA from mRNA of uninfected primary human myoctes in **figure 19** shows hybridizations to control genes (A10 to D11) in a similar pattern as seen before in the experiment with human muscle mRNA that had been purchased (**figure 13**). The same is true for the AICAR stimulated uninfected primary human myoctes hybridization results in **figure 20**.

There was no obvious difference in the hybridization patterns of those two hybridizations, i.e. AICAR stimulated and not-stimulated AMPK. Thus, AICAR did not lead to an observable alteration of gene expression due to AMPK stimulation.

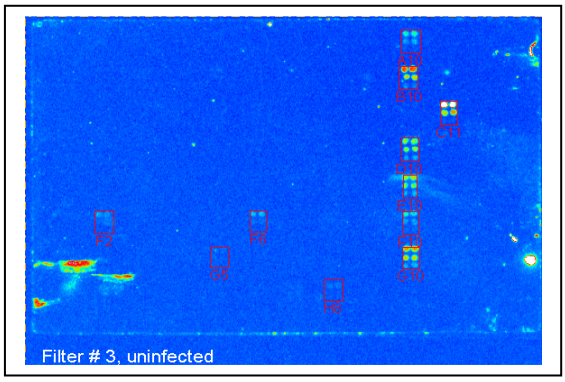


FIGURE 19: HYBRIDIZATION RESULT OF MRNA EXTRACT FROM UNINFECTED CELLS

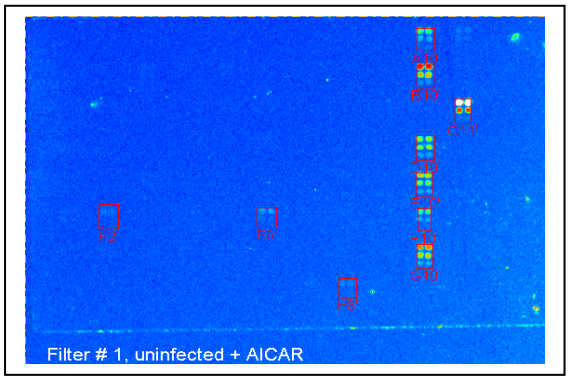


FIGURE 20: HYBRIDIZATION RESULT OF MRNA EXTRACT FROM UNINFECTED CELLS, STIMULATED WITH AICAR

AMPKα_2 adenovirus infected primary human myocytes with and without AICAR stimulation

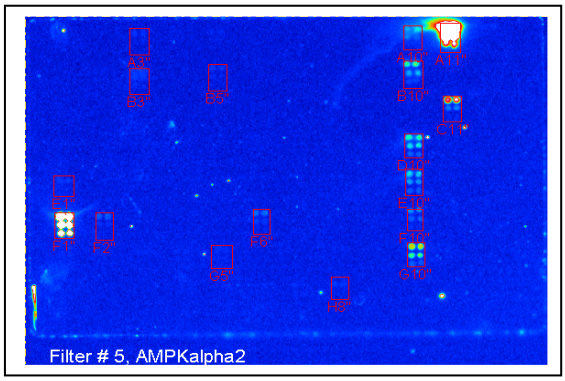


FIGURE 21: HYBRIDIZATION RESULT OF MRNA EXTRACT FROM AMPKα_2 INFECTED CELLS

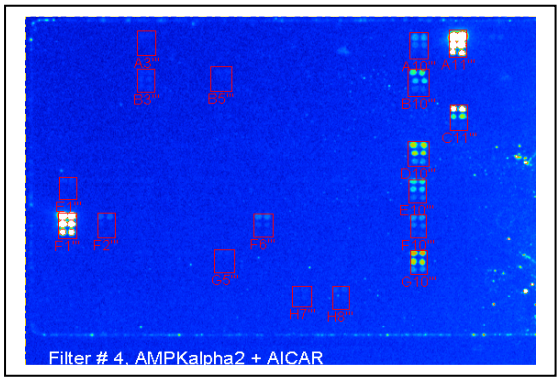


FIGURE 22: HYBRIDIZATION RESULT OF MRNA EXTRACT FROM AMPKα_2 INFECTED CELLS, STIMULATED WITH AICAR

As for uninfected primary human myocytes, there is no clear observable difference between AICAR stimulated cells and unstimulated infected cells (**figures 21 and 22**). However, two important differences in comparison to the uninfected controls is evident: GFP (A11) and AMPKα_2 (F1) were expressed and gave strong hybridization signals.

GFP adenovirus infected primary human myocytes with and without AICAR stimulation

Infection with a GFP adenovirus construct was carried out in order to compare this result with the AMPKα_2 recombinant adenovirus infected primary human myocytes. As can be seen in **figure 23 and 25** are the hybridization hits for GFP (A11) similar to those found on the filter arrays for AMPKα_2 (**fig. 21 and 22**). That indicates successful infection and coinciding expression of GFP.

Comparing AICAR stimulated GFP adenovirus infected cells with unstimulated results does not indicate important differences.

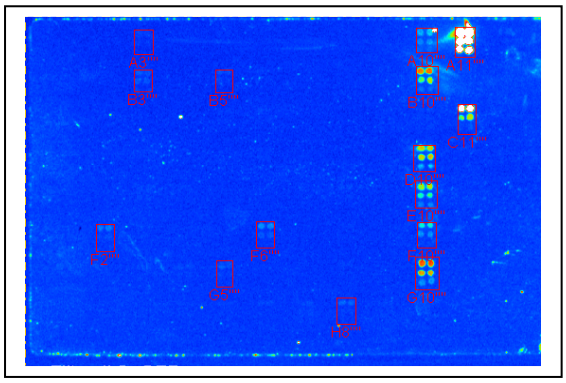


FIGURE 23: HYBRIDIZATION RESULT OF MRNA EXTRACT FROM GFP INFECTED CELLS

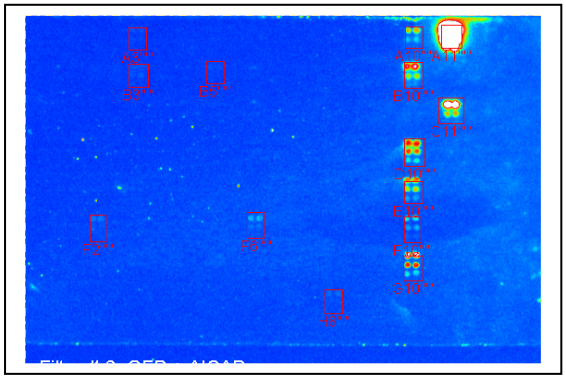


FIGURE 24: HYBRIDIZATION RESULT OF MRNA EXTRACT FROM GFP INFECTED CELLS, STIMULATED WITH AICAR

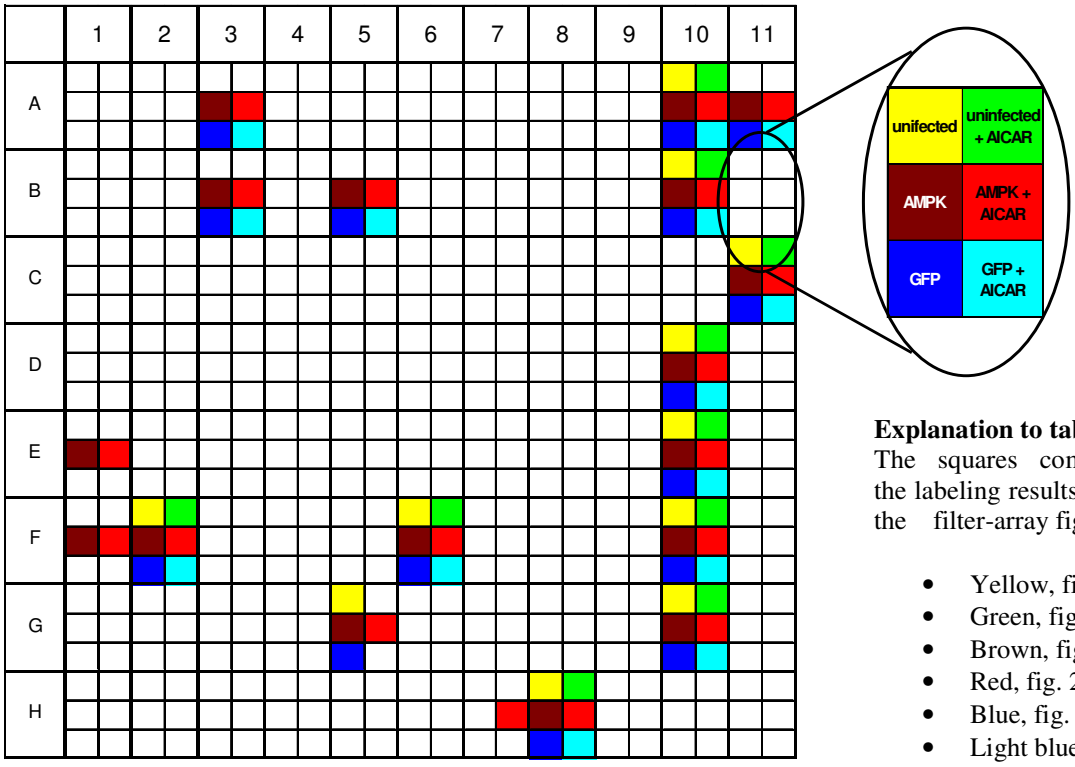
Comparison of the primary human myocytes hybridization results

The marked hybridization patterns are designated in accordance to the filter array gridding (**Fig. 11**). Most interestingly GFP expression is clearly visible for the mRNA extractions that were performed from infected cells (gene A11, **fig. 21 to 24**). That indicates that infection with recombinant adenovirus had been achieved. In **figures 21 and 22** a strong

signal for AMPKα_2 (gene F1) is visible. That is an indication that AMPKα_2 overexpression had been achieved. Therefore it appears that the technical aims of this project were fulfilled.

An overview of all the hits is shown in **table 11**. Here the squares stand for the different filter arrays (see explanation next to the table). The hybridization results for others than the control genes do not appear to give any indication of gene control due to AMPKα_2. In fact most of those signals are rather weak. As described before, this might be based on expression of those genes below the threshold of detection sensitivity of the filter. In order to determine the exact threshold of detection sensitivity one would have to include other control genes in the filter array, as described below. The results do not allow any conclusion concerning the research-oriented aspect of this exam work, i.e. to confirm the gene regulatory function of AMPK.

Table 11: Overview of mRNA extractions hybridization hits



Explanation to table 11:
The squares comprehend the labeling results (hits) in the filter-array figures:

- Yellow, fig. 19
- Green, fig. 20
- Brown, fig. 21
- Red, fig. 22
- Blue, fig. 23
- Light blue, fig. 24

Comparability of hybridization results

In order to be able to compare the different signals, the patterns of hybridization have to be standardized. This standardization, called "normalization", has to be done by comparison with the background, in order to avoid unspecific signals. Additionally the signals on a filter array have to be normalized with an independent "house-keeping gene", i.e. a not

regulated gene that has equal expression levels in all of the investigated cells. Only after this computational modification of the results, one can compare them and determine changes in expression. However, the signals must exceed a certain threshold in order to be suitable for analysis. As mentioned before, our signals were too weak to be used for such an analysis.

"House-keeping" genes should preferably be from plants when, as in these experiments, human mRNA is used (Cecile Martijn, personal contact). This "house-keeping" gene of a known concentration would have to be added to the labeling reaction. After hybridization the signal strength, produced by this known concentration of cDNA, could be compared with the other signals.

This was not the case with the control genes used in our filter array production. Therefore we could not determine the threshold of detection sensitivity. This improvement of the experiment could not be performed in the time frame of this exam work, but would have to be considered in future experiments (see chapter: "Future perspectives").

However, for GFP and AMPKα_2 it was possible to make a relative statement of gene expression. In case of GFP we were almost looking at an "on/off" situation of gene expression. That means that expression appeared to only occur in infected cells. Uninfected human primary myocytes do not express the GFP (**figures 21 to 24**).

Assuming that there was a basal expression, which should be the case in accordance to the literature[12], AMPKα_2 expression levels appeared to be too low to be detected. However, in comparison with the AMPKα_2 infected cells it was most obvious that overexpression had been successful, because there were clear signals (**figures 21 and 22**).

A further aspect of comparability was the usage of equal amounts of mRNA when labeling. In our experiment, all the extracted mRNA, i.e. varying amounts of mRNA were used. This was the case because an early labeling experiment (data not shown) had not given any result. Therefore all the mRNA was used, in order to determine the most efficient amount of mRNA to be used in future experiments. The downside of this obviously was that the intensity of signals between the filters was not comparable due to usage of varying amounts of labeled mRNA.

The conclusion of this experiment was that one would have to aim at considerably higher mRNA amounts for labeling and hybridization, in order to get sufficiently strong signals. That would most probably be around 3-5 µg of mRNA per labeling reaction as suggested by further experiments that meanwhile have been performed by Anna Krook's group at the Karolinska Institute (personal contact). Additionally "house-keeping" genes of a

different nature as the used control genes would have to be used and mRNA concentrations and ^{33}P incorporation be equalized, in order to get comparable results.

Conclusion

The final experiment performed in this study has to be considered as preliminary. Because of restricted time further experiments that would have to build on the gained experience could not be performed. Some techniques, used in this study, were too time consuming to be improved in the time frame of an exam-work. However, the performed experiments clearly indicated that the set up of the experiment was practicable for the objective, i.e. to investigate gene regulatory effects of AMPK.

Analysis of gene expression using gene filter arrays is a technique that has not yet been established as a standard protocol at Biovitrum. Working with the development and set up of such a protocol was a demanding and very interesting task. At the same time it is naturally rather time consuming to achieve functioning and reproducible results, applying a new technique. However, the main technical objective, i.e. to obtain a working protocol for gene expression analysis has been achieved.

The major research objective might not have been achieved to the full extend. Anyhow, the functionality of the filters has been demonstrated sufficiently. Therefore we are confident that future experiments will be successful in doing so (see "future perspectives" chapter for more details), and that using this technique, as set up by this exam work, some already published data will be confirmed. There also is a high probability of finding new targets of AMPKα2 gene expression regulation then.

Future perspectives

Confirmation of results

A thoroughly repetition of the hybridization experiments will be necessary using a significantly higher amount of mRNA, in order to confirm the results and to improve the experiment's conditions. Additionally the experiment will have to be repeated with mRNA extracts from several patients. This would rule out the possibility that the results are based on individuality.

Furthermore will the genes found to be regulated have to be confirmed as regulated in several ways. Firstly, DNA sequencing has to be performed to confirm that the fragments on the filter array consist of the desired unique sequence. Secondly, other techniques have to be applied, to confirm the actual result, e.g. Taqman PCR analysis. Thirdly protein extracts will have to be analyzed to make sure that the measured regulation patterns on the mRNA level are represented on the protein level as well. Assays for that would involve e.g. immuno-precipitation techniques and immuno blot analysis.

Continuing projects

Considering the broad involvement of AMPK in numerous pathways of the cell energy management, many more target enzymes have to be addressed for further analysis. Especially when it comes to AMPK's involvement in exercise induced metabolic changes questions remain unanswered.

In particular, the insulin independent AMPK induced glucose uptake into muscle cells is of interest concerning treatment of diabetes type II. Already today exercise is an established part of diabetes treatment. However, deeper insight into AMPK's role in cell metabolism after and during exercise as well as identification of new AMPK regulated genes may result in new strategies for diabetes treatment.

Using the techniques established in the exam work, these and related questions will be addressed in a doctoral thesis work which will start in 2002 at Karolinska Institutet, Stockholm.

Appendix

Agilent500DNA protocol measurement

BioSizing_DNA-500_00339_2002-01-24_14-32-56 Ladder

Peak	Mig.Time(secs)	Corr.Area	Size(bp)	Conc.(ng/ul)	Molarity(nmol/l)	Marker
1	38.85	30.50	15	4.2	424.24	Lower
2	41.10	31.00	25	2.0	121.21	
3	44.15	32.00	50	2.0	60.61	
4	48.70	35.00	100	2.0	30.30	
5	53.00	37.50	150	2.0	20.20	
6	57.70	40.00	200	2.0	15.15	
7	62.20	41.00	250	2.0	12.12	
8	66.65	41.50	300	2.0	10.10	
9	75.00	42.00	400	2.0	7.58	
10	80.85	42.00	500	2.0	6.06	
11	85.20	42.00	600	2.1	5.30	Upper

BioSizing_DNA-500_00339_2002-01-24_14-32-56 2e

Peak	Mig.Time(secs)	Corr.Area	Size(bp)	Conc.(ng/ul)	Molarity(nmol/l)	Marker
1	38.85	28.90	15	4.2	424.24	Lower
2	53.15	237.39	152	14.0	140.09	
3	85.20	37.86	600	2.1	5.30	Upper

BioSizing_DNA-500_00339_2002-01-24_14-32-56 2f

Peak	Mig.Time(secs)	Corr.Area	Size(bp)	Conc.(ng/ul)	Molarity(nmol/l)	Marker
1	38.85	30.43	15	4.2	424.24	Lower
2	53.25	168.90	153	9.7	95.89	
3	85.20	39.02	600	2.1	5.30	Upper

BioSizing_DNA-500_00339_2002-01-24_14-32-56 2g

Peak	Mig.Time(secs)	Corr.Area	Size(bp)	Conc.(ng/ul)	Molarity(nmol/l)	Marker
1	38.85	32.34	15	4.2	424.24	Lower
2	51.30	5.96	130	0.33	3.88	
3	52.45	184.08	144	10.1	106.84	
4	85.20	41.07	600	2.1	5.30	Upper

BioSizing_DNA-500_00339_2002-01-24_14-32-56 2h

Peak	Mig.Time(secs)	Corr.Area	Size(bp)	Conc.(ng/ul)	Molarity(nmol/l)	Marker
1	38.85	30.12	15	4.2	424.24	Lower
2	52.10	253.77	140	14.3	155.31	
3	85.20	40.31	600	2.1	5.30	Upper

BioSizing_DNA-500_00339_2002-01-24_14-32-56 3a

Peak	Mig.Time(secs)	Corr.Area	Size(bp)	Conc.(ng/ul)	Molarity(nmol/l)	Marker
1	38.85	30.55	15	4.2	424.24	Lower
2	52.95	165.36	149	8.6	87.51	
3	85.20	42.95	600	2.1	5.30	Upper

BioSizing_DNA-500_00339_2002-01-24_14-32-56 3b

Peak	Mig.Time(secs)	Corr.Area	Size(bp)	Conc.(ng/ul)	Molarity(nmol/l)	Marker
1	38.85	32.56	15	4.2	424.24	Lower
2	52.40	261.48	143	13.4	141.57	
3	85.20	44.24	600	2.1	5.30	Upper

BioSizing_DNA-500_00339_2002-01-24_14-32-56 3c

Peak	Mig.Time(secs)	Corr.Area	Size(bp)	Conc.(ng/ul)	Molarity(nmol/l)	Marker
1	38.85	29.59	15	4.2	424.24	Lower
2	52.80	252.42	148	14.5	149.13	
3	85.20	39.02	600	2.1	5.30	Upper

BioSizing_DNA-500_00339_2002-01-24_14-32-56 3d

Peak	Mig.Time(secs)	Corr.Area	Size(bp)	Conc.(ng/ul)	Molarity(nmol/l)	Marker
1	38.85	29.93	15	4.2	424.24	Lower
2	52.55	251.23	145	13.8	144.87	
3	85.20	40.94	600	2.1	5.30	Upper

BioSizing_DNA-500_00339_2002-01-24_14-32-56 3e

Peak	Mig.Time(secs)	Corr.Area	Size(bp)	Conc.(ng/ul)	Molarity(nmol/l)	Marker
1	38.85	30.00	15	4.2	424.24	Lower
2	52.80	216.22	148	13.0	133.87	
3	85.20	37.24	600	2.1	5.30	Upper

BioSizing_DNA-500_00339_2002-01-24_14-32-56 3f

Peak	Mig.Time(secs)	Corr.Area	Size(bp)	Conc.(ng/ul)	Molarity(nmol/l)	Marker
1	38.85	27.80	15	4.2	424.24	Lower
2	52.70	249.20	147	17.6	181.79	
3	85.20	31.90	600	2.1	5.30	Upper

BioSizing_DNA-500_00339_2002-01-24_14-32-56 3g

Peak	Mig.Time(secs)	Corr.Area	Size(bp)	Conc.(ng/ul)	Molarity(nmol/l)	Marker
1	38.85	28.78	15	4.2	424.24	Lower
2	52.75	215.40	147	14.7	151.62	
3	85.20	32.91	600	2.1	5.30	Upper

BioSizing_DNA-500_00339_2002-01-24_14-32-56 3h

Peak	Mig.Time(secs)	Corr.Area	Size(bp)	Conc.(ng/ul)	Molarity(nmol/l)	Marker
1	38.85	28.71	15	4.2	424.24	Lower
2	53.00	195.25	150	14.1	142.81	
3	85.20	30.94	600	2.1	5.30	Upper

Agilent6000nano RNA protocol measurement

BioSizing_mRNA-Nano_00339_2002-01-21_16-13-46 Ladder

Peak	Mig.Time(secs)	Corr.Area
1	19.60	7.28
2	24.90	20.54
3	28.15	14.32
4	31.55	7.49
5	35.25	16.60
6	40.30	30.90
7	44.30	13.04

RNA Area	130.82
RNA Concentration(ng/ul)	150.00

BioSizing_mRNA-Nano_00339_2002-01-21_16-13-46 ampk1

Peak	Mig.Time(secs)	Corr.Area
1	19.60	9.36
2	34.75	5.36
3	39.90	5.86
4	41.75	7.42

Fragment	Name	Start_Time(secs)	End_Time(secs)	Area	%_of_total_Area
1	rRNA	34.45	35.40	5.23	9.09
2	rRNA	41.50	42.05	7.07	12.28

RNA Area	57.57
RNA Concentration(ng/ul)	66.01
rRNA Contamination(%)	21.37

BioSizing_mRNA-Nano_00339_2002-01-21_16-13-46 2

Peak	Mig.Time(secs)	Corr.Area
1	19.60	8.93
2	34.90	3.56
3	40.05	3.73

Fragment	Name	Start_Time(secs)	End_Time(secs)	Area	%_of_total_Area

1	rRNA	34.60	35.50	3.37	11.22
2	rRNA	39.65	40.75	3.34	11.13

RNA Area 30.05
RNA Concentration(ng/ul) 34.45
rRNA Contamination(%) 22.35

BioSizing_mRNA-Nano_00339_2002-01-21_16-13-46 ampk-aicar1

Peak	Mig.Time(secs)	Corr.Area
1	19.60	9.17
2	34.80	4.04
3	39.90	6.75

Fragment	Name	Start_Time(secs)	End_Time(secs)	Area	%_of_total_Area
1	rRNA	34.50	35.35	3.81	7.12
2	rRNA	39.25	40.90	6.39	11.94

RNA Area 53.52
RNA Concentration(ng/ul) 61.37
rRNA Contamination(%) 19.06

BioSizing_mRNA-Nano_00339_2002-01-21_16-13-46 2

Peak	Mig.Time(secs)	Corr.Area
1	19.60	9.72
2	35.05	1.52
3	40.25	1.90

Fragment	Name	Start_Time(secs)	End_Time(secs)	Area	%_of_total_Area
1	rRNA	34.80	35.55	1.38	6.58
2	rRNA	39.85	40.90	1.64	7.82

RNA Area 20.92
RNA Concentration(ng/ul) 23.99
rRNA Contamination(%) 14.41

BioSizing_mRNA-Nano_00339_2002-01-21_16-13-46 gfp1

Peak	Mig.Time(secs)	Corr.Area
1	19.60	9.49
2	34.75	5.81
3	39.95	6.22

Fragment	Name	Start_Time(secs)	End_Time(secs)	Area	%_of_total_Area
1	rRNA	34.45	35.30	5.74	12.03
2	rRNA	39.40	40.70	5.77	12.08

RNA Area 47.73
RNA Concentration(ng/ul) 54.74
rRNA Contamination(%) 24.10

BioSizing_mRNA-Nano_00339_2002-01-21_16-13-46 2

Peak	Mig.Time(secs)	Corr.Area
1	19.60	9.60
2	34.70	5.62
3	39.85	6.31

Fragment	Name	Start_Time(secs)	End_Time(secs)	Area	%_of_total_Area
1	rRNA	34.40	35.25	5.33	13.45
2	rRNA	39.15	40.80	5.92	14.95

RNA Area	39.62
RNA Concentration(ng/ul)	45.43
rRNA Contamination(%)	28.40

BioSizing_mRNA-Nano_00339_2002-01-21_16-13-46 gfp-aicar1

Peak	Mig.Time(secs)	Corr.Area
1	19.60	8.19
2	34.70	4.05
3	39.80	4.28

Fragment	Name	Start_Time(secs)	End_Time(secs)	Area	%_of_total_Area
1	rRNA	34.40	35.25	3.92	11.61
2	rRNA	39.25	40.60	3.95	11.70

RNA Area	33.76
RNA Concentration(ng/ul)	38.71
rRNA Contamination(%)	23.31

BioSizing_mRNA-Nano_00339_2002-01-21_16-13-46 2

Peak	Mig.Time(secs)	Corr.Area
1	19.60	8.03
2	34.75	2.37
3	39.75	2.38

Fragment	Name	Start_Time(secs)	End_Time(secs)	Area	%_of_total_Area
1	rRNA	34.45	35.30	2.25	8.27
2	rRNA	39.30	40.45	2.01	7.39

RNA Area	27.17
RNA Concentration(ng/ul)	31.15
rRNA Contamination(%)	15.66

BioSizing_mRNA-Nano_00339_2002-01-21_16-13-46 no.inf1

Peak	Mig.Time(secs)	Corr.Area
1	19.60	8.59
2	34.55	4.37
3	39.60	3.19

Fragment	Name	Start_Time(secs)	End_Time(secs)	Area	%_of_total_Area
1	rRNA	34.30	35.15	4.18	10.47
2	rRNA	39.00	40.25	2.73	6.83

RNA Area	39.94
RNA Concentration(ng/ul)	45.79
rRNA Contamination(%)	17.30

BioSizing_mRNA-Nano_00339_2002-01-21_16-13-46 2

Peak	Mig.Time(secs)	Corr.Area
1	19.60	8.16
2	34.65	1.94
3	39.60	1.59

Fragment	Name	Start_Time(secs)	End_Time(secs)	Area	%_of_total_Area
1	rRNA	34.35	35.25	1.87	5.51
2	rRNA	39.20	40.20	1.24	3.67

RNA Area	33.94
RNA Concentration(ng/ul)	38.92
rRNA Contamination(%)	9.18

BioSizing_mRNA-Nano_00339_2002-01-21_16-13-46 no.inf.-aicar1

Peak	Mig.Time(secs)	Corr.Area
1	19.60	7.08
2	34.70	1.69
3	39.55	1.06

Fragment	Name	Start_Time(secs)	End_Time(secs)	Area	%_of_total_Area
1	rRNA	34.35	35.30	1.56	18.82
2	rRNA	39.20	40.15	0.90	10.87

RNA Area	8.27
RNA Concentration(ng/ul)	9.48
rRNA Contamination(%)	29.69

BioSizing_mRNA-Nano_00339_2002-01-21_16-13-46 2

Peak	Mig.Time(secs)	Corr.Area
1	19.60	8.25
2	34.60	1.50
3	39.50	1.42

Fragment	Name	Start_Time(secs)	End_Time(secs)	Area	%_of_total_Area
1	rRNA	34.35	35.05	1.44	4.52
2	rRNA	39.10	40.20	1.19	3.73

RNA Area	31.86
RNA Concentration(ng/ul)	36.53
rRNA Contamination(%)	8.25

References

[1] **D. Krämer**
Internship-Report, Biovitrum: production of a recombinant adenovirus-construct
Unpublished report for the University of applied science, Aachen, Germany, 2001

[2] **Tong-Chuan He, Shibin Zhou, Luis T. Da Costa, Jian Yu, Kenneth W. Kinzler, and Bert Vogelstein**
A simplified system for generating recombinant adenoviruses
Proc. Natl. Acad. Sci. USA, Vol. 95, pp. 2509-2514, March 1998, Medical sciences

[3] **Peter C. F. Cheung, Ian P. Salt, Stephen P. Davies, D. Grahame Hardie and David Carling**
Characterization of AMP-activated protein kinase gamma-subunit isoforms and their role in AMP binding
Biochemical Society 2000, 346, 659-669

[4] **James Mu, Joseph T. Brozinick Jr., Otto Valladares, Maja Bucan, and Morris J. Birnbaum**
A role for AMP-activated protein kinase in contration- and hypoxia-regulated Glucose transport in skeletal muscle
Molecular Cell, Vol. 7, 1085-1094, May 2001

[5] **Stumvoll M**
Pathogenesis of type 2 diabetes mellitus
B.I.F. Futura Vol.13 (1998)

[6] **Lodish H., Baltimore D., Berk A., Zipursky S.L., Matsdaira P., Darnell J.**
Moleclar Cell Biology, 3rd edition (1995),
W. H. Freeman and Company, New York, Page 911

[7] **Rhoades R.R., Tanner G.A.**
Medical Physiology, 1st edition (1995),
Lippiscott Williams & Wilkins, Baltimore, Pages 715-719

[8] **D. Grahame Hardie, David Carling, and Marian Carlson**
The AMP-activated/SNF1 protein kinase subfamily: metabolic sensor of the eukaryotic cell?
Annu. Rev. Biochem. 1998. 67:821-55

[9] **David Carling, Paul R. Clarke, Victor A. Zammit and D. Grahame Hardie**
Purification and characterization of the AMP-activated protein kinase
Eur. J. Biochem 186, 129-136 (1998)

[10] **CheungPCF, Salt IP, Davies SP, Hardie DG, Carling D.**
Characterization of AMP-activated protein kinase γ-subunit isoforms and their role in AMP binding
Biochemical Society 2000, 346, 659-669

[11] **Winder WW.**
Energy-sensing and signaling by AMP-activated Protein kinase in skeletal muscle
J Appl. Physiol. 91: 1017-1028, 2001

[12] **Bruce E. Kamp, Ken I. Mitchelhill, David Stapleton, Belinda J. Michell, Zhi-Ping Chen and Lee A. Witters**
Dealing with energy demand: the AMP-activated protein kinase
TIBS 24 Jan. 1999, 22-25

[13] **D. Grahame Hardie**
Metabolic control: a new solution to an old problem
Current Biology 2000, 10:R757-759

[14] **Lee A Witters, Guang Gao, Bruce E. Kemp and Björn Quistorff**
Hepatic 5'-AMP-activated protein kinase: zonal distribution and relationship to Acetyl-CoA carboxylase activity in varying nutritional states
Archives of biochemistry and biophysics, Vol. 308, No. 2, Feb. 1, pp. 413-419, 1994

[15] **G. F. Merrill, E. J. Kurth, D. G. Hardie, and W. W. Winder**
AICA riboside increases AMP-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle
The American Physiological Society, Sep. 4, E1107-E1111

[16] **Marc Foretz, David Carling, Colette Guichard, Pascal Ferré, and Fabienne Foufelle**
AMP-activated protein kinase inhibits the glucose-activated expression of fatty acid synthase gene in rat hepatocytes
The journal of biological chemistry,Vol. 273, No. 24, Issue June 12, pp. 14767-14771, 1998

[17] **Isabelle Leclerc, Axel Kahn, Bruno Doiron**
The 5'-AMP-activated protein kinase inhibits the transcriptional stimulation by glucose in liver cells, acting through the glucose response complex
FEBS letters 431 (1998) 180-184

[18] **Demetrios Vavvas, Alexios Apazidis, Asish K. Saha, James Gamble, Abhay Patel, Bruce E. Kamp, Lee A. Witters, and Neil B. Ruderman**
Contraction-induced changes in acetyl-CoA carboxylase and 5'-AMP-activated kinase in skeletal muscle
JBC, Vol. 272, No. 20, Issue May 16, pp. 13255-13261, 1997

[19] **Tatsuya Hayashi, Jörgen F. P. Wojtaszewski and Laurie J. Goodyear**
Exercise regulation of glucose transport in sceletal muscle
The American Physiological Society, 1997, E1039-E1051

[20] **Jörgen F.P. Wojtaszewski, Yasuki Higaki, Michael F. Hirshman, M. Dodson Michael, Scott D. Dufresne, C. Ronald Kahn, and Laurie J. Goodyear**
Exercise modulates postreceptor insulin signaling and glucose transport in muscle-specific insulin receptor knock-out mice
Journal of Clinical Investigation Nov. 1999, Vol. 104, No. 9, 1257-1264

[21] **Raynald Bergeron, Raymond R. Russell III, Lawrence H. Young, Jian-Ming Ren, Melissa Marcucci, Agnes Lee, and Gerald I. Shulman**
Effect of AMPK activation on muscle glucose metabolism in conscious rats
The American Physiological Society Jan. 1999, E938-E944

[22] **Gabriela da Silvia Xavier, Isabelle Leclerc, Ian P. Salt, Bruno Doiron, D. Grahame Hardie, Axel Kahn, and Guy A. Rutter**
Role of AMP-activated protein kinase in the regulation by glucose of islet beta cell gene expression
PNAS April 11 ,2000, Vol. 97, No. 8, 4023-4028

[23] **Pamela A. Lochhead, Ian P. Salt, Kay S. Walker, D. Grahame Hardie, and Calum Sutherland**
5-Aminoimidazole-4-Carboxamide riboside mimics the effects of insulin on the expression of the 2 key gluconeogenic genes PEPCK and Glucose-6-Phosphatase
Diabetes, June 2000, Vol. 49, 896-903

[24] **Deborah M. Muoio, Kimberly Seefeld, Lee A. Witters and Rosalind A. Coleman**
AMP-activated kinase reciprocally regulates triacylglycerol sythesis and fatty acid oxidation in liver and muscle: evidence that sn-glycerol-3-phosphate acyltransferase is a novel target
Biochem. J, 1999 338, 738-791

[25] **Sören N. Jakobsen, D. Grahame Hardie, Nick Morrice, Hans E. Tornqvist**
AMPK phosphorylates IRS-1 on Ser789 in mouse C2C12 myotubes in response to 5-aminoimidazole-4-carboxamide riboside
The American Society for Biochemistry and Molecular Biology, 2001 (published as manuscript C100483200 in Oct. 2001)

[26] **Scott M. Warden, Christine Richardson, John O'Donnell Jr, David Stapleton, Bruce E. Kemp and Lee A. Witters**
Post-translational modifications of the beta-1 subunit of AMP-activated protein kinase affect enzyme activity and cellular localization
Biochem. J. (2001) 354, 275-283

[27] **Buhl, Esben S.; Jessen, Niels; Schmitz, Ole; Pedersen, Steen B.; Pedersen, Oluf; Holman, Geoffrey D.; Lund, Sten**
Chronic treatment with 5-aminoimidazole-4-carboxamide-1-[beta]-D-ribofuranoside increases Insulin-stimulated glucose uptake and glut4 translocation in Rat skeletal muscle in a fiber Type-specific manner
Diabetes, Vol. 50(1). Jan. 2001.12-17

[28] **Donghai Zheng, Paul S. MacLean, Steven C. Pohnert, John B. Knight, Ann Louise Olson, William W. Winder, and G. Lynis Dohm**
Regulation of muscle GLUT-4 transcription by AMP-activated protein kinase
J Appl Physiol 91: 1073-1083, 2001

[29] **Xia Xi, Jianhuai Han, and Jin-Zhong Zhang**
Stimulation of glucose transport by AMP-activated protein kinase via activation of p38 mitogen-activated protein kinase
The Journal of Biological Chemistry, Vol. 276, No. 44, Issue of Nov. 2, pp. 41029-41034, 2001

[30] **Wafa Abbud, Sue Habinowski, Jin-Zhong Zhang, Julie Kendrew, Fadel S. Elkairi, Bruce E. Kemp, Lee A. Witters, and Faramarz Ismail-Beigi**
Stimulation of AMP-activated protein kinase (AMPK) is associated with enhancement of Glut1-mediated glucose transport
Archives of Biochemistry and Biophysics, Aug. 15, 2000, Vol. 380, pp. 347-352

[31] **M. F. White**
The insulin signalling system and the IRS proteins
Diabetologia (1997) 40:S2-S17

[32] **Lodish H., Baltimore D., Berk A., Zipursky S.L., Matsdaira P., Darnell J.**
Moleclar Cell Biology, 3rd edition (1995)
W. H. Freeman and Company, New York, pages 907-910

[33] **Internet resource**: http://www.queensu.ca/micr/micr450/adeno.html

[34] **Internet resource**: http://www-micro.msb.le.ac.uk/335/Adenoviruses.html

[35] **Ronald P. Leon, Tammy Hedlund, Sandra J. Meech, Shaobing Li, Jerome Schaacks, Stephen P. Hunger, Richard C. Duke, and James DeGregori**
Adenoviral-mediated gene transfer in lymphocytes
PNAS, Vol. 95, pp. 13159-13164, October 1998, Immunology

[36] **Walter Doerfler**
Adenoviruses – paradigms for mammalian biology
B.I.F. Futura, Vol. 17 (2002)

[37] **Mary Hitt, Andrew Bett, Ludvik Prevec, and Frank L. Graham**
Cell biology: a laboratory handbook; Construction and propagation of human adenovirus vectors,
Academic press 1998, 2nd edition Vol. 1, pages 500ff

[38] **Magdalena Gabig and Grzegorz Wegrzyn**
An introduction to DNA chips: principles, technology, applications and analysis
Acta Biochimica Polonica Vol. 48 No. 3/2001, 615-622

[39] **Xinmin Li, Weikun Gu, Subburuman Mohan, and David J. Baylink**
DNA Microarrays, their use and misuse
Microcirculation (2002) 9, 13-22, Nature publishing group

[40] **John C. Rockett, J. Christopher Luft, J. Brian Garges, Stephen A. Krawetz, Mark R. Hughes, Kwan Hee Kim, Asa J. Oudes and David J. Dix**
Development of a 950-gene DNA array for examining gene expression patterns in mouse testis
Genome Biology 2001, 2(4): research0014.1-0014.9

[41] **GenBank database**: http://www.psc.edu/general/software/packages/genbank/genbank.html

[42] **UniGene database:** http://www.ncbi.nlm.nih.gov/UniGene/

[43] **D.T Suzuki, A.J.F. Griffiths, J.H. Miller, R.C. Lewontin**
Genetik, 1st edition (German) 1991
VCH Verlagsgesellschaft mbH, Bundesrepublik Deutschland, Chapter 16, page 389ff