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Gene Expression of Human T Lymphocytes Cell Cycle: Experimental and Bioinformatic Analysis

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Abstract Human lymphocytes gene expression is monitored before and after PHA stimulation over 72 h, using DNA microarray technology. Results are then compared with our previous bioinformatics predictions, which identified six leader genes of highest importance in human T lymphocytes cell cycle. Experimental data are strikingly compatible with bioinformatic predictions of the specific role and interaction of PCNA, CDC2, and CCNA2 at all phases of the cell cycle and of CHEK1 in regulating DNA repair and preservation. It does not escape our notice that the conception and use of ad hoc arrays, based on a bioinformatics prediction which identifies the most important genes involved in a particular biological process, can really be an added value in cell biology and cancer research alternative to massive frequently misleading molecular genomics. J. Cell. Biochem. 99: 1326–1333, 2006. © 2006 Wiley-Liss, Inc.

Key words: lymphocytes; PHA stimulation; bioinformatics; gene microarray

DNA microarrays have emerged as one of the most promising methods for the analysis of gene expression [Butte, 2002; Nicolini et al., 2006]. This technique allows the study of an immense amount of genes (over 10,000) with only one experiment and therefore can draw a picture of a whole genome. Anyway, the huge number of data coming out from microarray experiments may often raise experimental complications and difficulties in the analysis. Moreover, the greatest part of genes displayed on an array is often not directly involved in the cellular process being studied. Commercial arrays with a lower number of genes—usually of 150–200—are currently available, but the genes displayed are usually once again chosen without a precise consideration of the particular target of the study.

Recently, we proposed a bioinformatics algorithm, based on the scoring of importance of genes and a subsequent cluster analysis, which allowed us to determine the most important genes, that we call "leader genes" [Sivozhelezov et al., 2006] in human T lymphocytes cell cycle. The basis of the scoring system relies upon the calculation of interactions among genes, performed with software available in the web, such as STRING [von Mering et al., 2005]. The number of links for each gene is then weighted and the final weighted numbers of links are clustered, in order to make a hierarchical classification of genes [Sivozhelezov et al., 2006]. In this way, it becomes possible to draw and to update maps of the major biological control systems, and to integrate them in a concise manner to discern common patterns of interactions between gene expression and their correlated coding of proteins. This work intends to confirm our earlier suggestion [Nicolini et al., 2006; Sivozhelezov et al., 2006] that the "leader genes" identification is directly related to the control of gene expression and is potentially applicable to all investigation of gene expressions. Namely as suggested explicitly in [Nicolini et al., 2006] the construction of dedicated microarrays using known leader genes will be important for all investigators in the field of cell biology and gene expression.

We chose, as a model system, human T cell lymphocytes stimulated to entry cell cycle with PHA [Nicolini et al., 2006]. This particular cellular system is very well known and was quantitatively characterized time ago [Abraham et al., 1980; Oosterwegel et al., 1999; Cantrell, 2002;

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Isakov and Altman, 2002]; therefore, it can be a good starting point to verify our algorithm. In particular, we identified 238 genes involved in the control of cell cycle. Most important, only six of them were previously identified to be the leader genes as shown in Table 4 of Sivozhelezov et al., 2006. Interestingly, the same six leader genes are involved in the cell cycle control at important progression points, namely the most important four at the transition from G0 to G1 phase [MYC; Oster et al., 2002], at the progression in G1 phase [CDK4; Modiano et al., 2000], and at the transitions from G1 to S [CDK2; Kawabe et al., 2002], and from G2 to M phases [CDC2; Baluchamy et al., 2003; Torgler et al., 2004]. The two remaining "leader genes" (CDKN1A and CDKN1B) are inhibitors of cyclin-CDK2 or -CDK4 complexes and thereby contribute to the control of G1/S transition and of G1 progression [Jerry et al., 2002; Chang et al., 2004].

We also confirmed our results by analyzing changes in gene expression after 48 h, using a newly developed and simple technology called DNASER, which is a novel bioinstrumentation for real-time acquisition and elaboration of images from fluorescent DNA microarrays developed in our laboratories [Nicolini et al., 2002, Troitsky et al., 2002]. The validity of the DNASER measurements was confirmed by standard fluorescence microscopy equipped with CCD. This experimental analysis proved that DNASER is appropriate for monitoring gene expression during the human lymphocytes cell cycle [Nicolini et al., 2006].

The leader gene approach, validated by experimental analysis on a model system, can suggest a more rationale approach to experimental techniques and methods, as DNA microarray. The application of bioinformatics studies and the identification of leader genes can predict the most important genes in a particular cellular process. In this way, it becomes possible to design smaller microarrays, which display only the most interesting genes for a specific cellular process and thus are much easier to interpret. Protein microarrays are also used for the study of protein–protein and protein–gene interactions [Ramachandran et al., 2004]. Like for the DNA microarrays, the leader gene approach can simplify their analysis, by reducing the protein displayed to the most important ones to be subsequently tested by mass-spectrometry or by ad hoc experimentation.

In this article, we experimentally analyze the gene expression of human T lymphocytes treated with the mitogen compound PHA 24, 48, and 72 h ['time series analysis' for a complete reference see Straume, 2004; Willbrand et al., 2005] after the stimulation and compare the results with independent bioinformatics predictions, in order to give a further validation to leader gene approach and to identify co-expressions among genes involved in the cell cycle of human T lymphocytes.

MATERIALS AND METHODS

All reagents and materials were purchased from Sigma Aldrich (St. Louis, MO) if not otherwise specified.

Cell Systems

As previously shown [Nicolini et al., 2006], to obtain human lymphocytes a sample of heparinized peripheral blood specimens was diluted 1:1 with sterile phosphate buffer saline (PBS, pH 7.2). The sample was centrifuged on a Ficoll-Hypaque gradient (specific gravity 1.080) at 1,500 rpm for 40 min at room temperature without brake [Abraham et al., 1980]. The lymphocytes rich interface was collected and washed twice with PBS, and the number of cells counted in presence of trypan blue. The 2×10^6 cells were seeded into each well containing 2 ml of RPMI 1,640 culture medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin 100 U/ml and streptomycin 50 μg/ml. The cells has then been incubated at 37°C in a humid chamber with 5% of CO_2 for 24, 48, 72 h. The cells have been then collected and counted and then it has been proceeded to RNA extraction.

cDNA Syntesis and Microarray Hybridization

As previously shown [Nicolini et al., 2006] the CyScriptRT enzyme, together to 25 μg of RNA (for each sample) and to dCTP Cy3 and dCTP Cy5 nucleotides, has been employed in a synthesis reaction lead to 42°C for 1 h and ½ in order to obtain the labeling by retrotranscription. The synthesis product of this reaction, marked cDNA, has been then subordinate to a purification step. Total RNA was extracted and amplified using T-7 in vitro transcription. The cDNA marked samples have been purified employing a purification kit and chromatographic columns supplied by Amersham Biosciences. The cDNA

obtained has been precipitated and resuspended in bidistilled water to quantify, by spectrophotometry, the samples and to verify the labeling. The cDNA marked samples have been subsequently lyophilized and resuspended in an opportune volume (120 μl) of hybridization buffer (Salt-Based hybridization). For the array hybridization 1 μg of cDNA marked with Cy3 and 1 μg of cDNA marked with Cy5 they have been mixed in a tube and resuspended in the hybridization buffer together to the control sample (Arabidopsis control). The sample thus obtained has been denatured and spotted on the array. The matrices have been then put in a hybridization chamber at 42°C for 20 h. To eliminate the aspecific binding the array has been washed with SSC buffers of decreasing concentration.

Human Starter Array

The employed array is the Human Starter Array by MWG Biotech, chosen on the base of the gene, we are interested about [Nicolini et al., 2006]. It contains 161 oligonucleotides (designed to be specific for the respective human gene sequence), 32 replicas and 7 gene specific Arabidopsis control oligonucleotides, for 200 total spots, disposed in 10 columns and 20 rows, more an exact copy, in total 400 spots for Array. The diameter of one spot is 100 μm and the distance between two near spots is 250 μm.

Fluorescence Microscopy

As previously shown [Nicolini et al., 2006] to test the results obtained by DNASER technology the microarray was analyzed by fluorescence microscopy; the analysis was lead with an Axioplan Zeiss microscope equipped with an objective of magnification 2× (in order to acquire the greatest possible area) and Zeiss filter sets no 15 and 26, chosen to visualize the Cy3 and Cy5 dye, respectively. Digital pictures were obtained by an air-cooled scientific grade CCD camera Zeiss, with a dynamic range of 14 bits (gray levels from 0 to 16,383). The obtained images were analyzed by GenePix software. We compared the gene expression of resting cells ($t = 0$), with the ones 24, 48, and 72 h after the PHA stimulation.

Extraction of the Total RNA and Labeling

As previously shown [Nicolini et al., 2006], in order to obtain total RNA extraction the cells pellet (minimal 1×10^7 cells) has been dealt with an extraction kit from Amersham Biosciences containing LiCl, CsTFA and an extraction buffer. The samples thus obtained have been conserved at −80°C. For the estimation of the extracted RNA they have been used 200 μl RNAsi free cuvettes. In order to avoid contaminations of genomic DNA, the RNA samples have been subordinates to digestion with the enzyme Dnase I.

RESULTS AND DISCUSSION

For every lymphocyte stimulation of the time sequence here reported, we obtained a good RNA total extraction yield as can be seen in Table I: the spectrophotometrical analysis has evidenced indeed a high RNA purity degree, being the ratio 260/280 nm always more than 1.9.

In Table II 32 genes were identified to be expressed during the 72 h of analysis, 8 of them (25%) were included in the list of 238 genes involved in the control of human T lymphocytes cell cycle [Sivozhelezov et al., 2006]. Among the 238 genes, we previously identified the six "leader genes," that is, those showing the highest number of interactions with other genes [Sivozhelezov et al., 2006]. Three of them (MYC, CDC2, CDK4) were present on the Starter Array and, as a confirmation of our prediction, they were all expressed during different experiments. For instance, MYC is the gene with the

TABLE I. RNA Extraction Yield for the Different Lymphocytes Samples

Time, in hours, after PHA stimulation	Number of cells	% of quiescent cells (in G0 + Q)	% of proliferant cells (in G1, S, G2)	Total RNA pg/cell	Fluorochromes utilized
0	10^7	90	8, 1, 1	30	Cy3 green
24	5×10^6	53	51, 8, 3	30, 4 (35, 7 %)	Cy5 red
48	5×10^6	36	—	39, 4 (+60 %)	Cy5 red
72	5×10^6	17	45, 36, 2	38, 4 (+72 %)	Cy5 red

TABLE II. Gene Expression of Human T Lymphocytes Stimulated With PHA, Derived Form Microarray Analysis in Different Times After the Stimulation

GENE	0 h	24 h	48 h	72 h	House-keeping	Present in 238 genes	Weighed number of links	Class
ADPRT	Middle			Middle				
ALDOA			Low					
ALPHA TUBULIN	Middle	Middle	Middle	Middle	X			
CCNA2		Middle		Middle		X	15.67	B
CCNE1		High	Low	Low		X	12.12	C
CCNG1		Low						
CCNH		High	High			X	11.37	C
CDC2		Low		Middle		X	26.47	A
CDK4		Middle	Low			X	25.25	A
CHEK1		Middle		Middle		X	9.35	D
EEF2				Middle				
FOS		Low						
GAPDH		Middle	Low	Middle	X			
MAD2L1		Low	Low	Low	X			
MYC		Low	Low			X	27.80	A
PCNA		Middle		High		X	17.59	B
PFDN4			Middle	Middle				
PFN1			Low					
POU2F2	Low			High				
RAB33A			Low					
RFC2				Low				
RPL10	Low	High	Middle	Low	X			
RPL11	High							
RPL16		Middle						
RPL18								
RPL32				High				
RPL8			Low					
TGFA			Low	Middle				
TOP1				Middle				
UBIQUITIN	Low	Middle	Middle	Middle	X			
UBL1 (SUMO)	Low	Low						
XRCC5		Low	Low	Low	X			
Total	7	18	16	19	6	8		

Intensities are normalized. Low: intensity between 0.1 and 0.33. Middle intensity between 0.34 and 0.66; high intensity >0.66. Genes belonging to the theoretically predicted list of 238 genes important in human T lymphocytes cell cycle are pointed out, marking also their class of importance, that is, leader, class B, C, or D [Sivozhelezov et al., 2006].

highest number of interaction in our bioinformatic predictions. It is known as a very early gene in the proliferative response, since it regulates the entrance in G1 phase of the cell cycle [Oster et al., 2002]. It is interesting to notice that MYC is expressed, in smaller quantities, after 24 and 48 h. Moreover, the absolute intensity of the corresponding spot on the array decreased during time, as expected from the entrance in the cell cycle and the progression along it. The two other "leader genes" are CDK4 and CDC2. The former is known to regulate the progression in G1 phase, while the latter is involved in the G1/S and G2/M transition [Modiano et al., 2000; Kawabe et al., 2002; Baluchamy et al., 2003]. Comfortingly CDK4 reaches its maximum expressed after 24 h and it is lower after 48 h. After 72 h, the corresponding spot cannot be identified on the array. This can points that, after 24 h, human T lymphocytes have entered the cell cycle (MYC and CDK4 expression) and are progressing along the G1 phase. CDC2 encodes for a member of the Ser/Thr protein kinase family. This protein is a catalytic subunit of the highly conserved protein kinase complex known as M-phase promoting factor (MPF), which is essential for G1/S and G2/M phase transitions of eukaryotic cell cycle [Kawabe et al., 2002; Baluchamy et al., 2003]. Mitotic cyclins stably associate with this protein and function as regulatory subunits [Olashaw and Pledger, 2002]. This gene regulates the progression from G1 to S and from G2 to M phase, and therefore represents an important signal of cell cycle progression [Modiano et al., 2000]. Its expression varies during cell cycle, as shown by our results: it is indeed not expressed in resting cells and it starts to be expressed only after 24 h. The expression decreases, so that cannot be identified, after 48 h and then it is already detectable at the end of the experiments. These results and those derived by MYC and CDK4 expression are compatible with the expectations: 24 h after a mitogen stimulation with PHA resting T lymphocytes have started cell cycle (MYC),

progressed along G1 phase (CDK4) and are preparing to replicate DNA (CDC2). Also, after 72 h, T lymphocytes are about to enter mitosis (CDC2). The bioinformatic-based identification of leader genes as most important genes for each of the progression points in cell cycle is thus perfectly confirmed.

Our prediction identified also other genes whose importance was not as high as "leader gene," but slightly lower. In fact, we previously identified "leader genes" using clustering techniques [Sivozhelezov et al., 2006]. The "leader genes" were selected as the gene belonging to the cluster with the highest numbers of links (class A), but there were also other important genes in other high-ranked classes emerging from the clustering process (classes B, C, and D, with decreasing importance). Of the eight genes identified on the array and included in the list of Sivozhelezov et al. [2006], three were of class A (leader genes), two of class B, two of class C, and only one of class D (Table II). For instance, the gene encoding for proliferating cell nuclear antigen, PCNA, belongs to class B (weighed number of links = 17.59). The protein encoded by this gene is an auxiliary protein of DNA polymerase delta and appears to be requested for both DNA synthesis and DNA repair [Ohta et al., 2002]. This gene is present in low amount in resting normal human T lymphocytes and, upon mitogen stimulation, begins to increase in mid-G1 phase, approximately 12–15 h before entry into S phase [Ohta et al., 2002]. PCNA continues to increase in amount throughout the cell cycle and remains high in proliferating cultures. This agrees with our experimental data shown in Table II. In fact, at the baseline we did not identify PCNA to be expressed on the array. After 24 h, PCNA expression has increased and it reaches its maximum level after 72 h. Interestingly, we were not able to identify its expression after 48 h: this seems in contrast with the considerations reported above, but probably the missed identification may be due to experimental problems. This addressed our attention to have a deeper view of the behavior of this gene, using also bioinformatics resources.

We used the on-line available software STRING [von Mering et al., 2005] to formulate a detailed prediction of PCNA interactions with other identified genes, not considering text-based interactions. The results are shown in Figure 1. PCNA is in the center of a complex

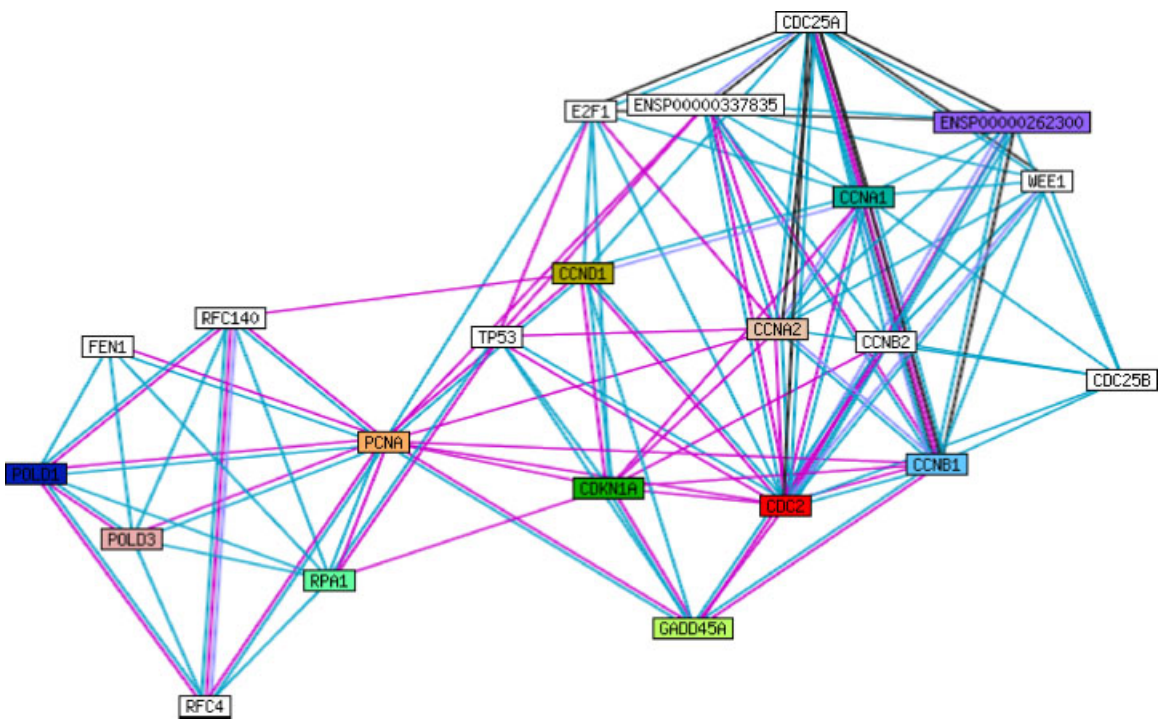

Fig. 1. Predicted gene interactions for PCNA and CDC2 and their neighborhood, according to STRING [von Mering et al., 2005]. The lines which connect single genes represent physical interaction between proteins, confirmed by various experimental methods, or the involvement in the same metabolic pathway.

map of interactions and is involved in many biochemical pathways. These interactions can also be identified from our experimental data. For instance, an interaction was predicted between PCNA and RFC2, a monomer of a heteropentameric protein complex consisting of the Rfc1, Rfc2, Rfc3, Rfc4, and Rfc5 subunits [Majka and Burgers, 2004; Majka et al., 2004]. This interaction is confirmed by our experimental data, which shows that RFC2 has a great level of expression after 72 h. Also, other predicted interactions between PCNA and other identified genes confirm our experimental data, such as the co-expression with CCNA2 [Vendrell et al., 2004]. This protein was identified by us to be a class B gene for the control of cell cycle in human T lymphocytes [Sivozhelezov et al., 2006] (weighed number of links = 15.67). The protein encoded by this gene belongs to the highly conserved cycling family, whose members vary in protein abundance through the cell cycle and act as regulators of CDK kinases. This cycling binds and activates CDC2 and thus promotes G1/S and G2/M transitions along the cell cycle [Vendrell et al., 2004]. PCNA, CCNA2, and CDC2, which are genes of great importance in human T lymphocytes cell cycle, are thus bound by a very close link, which is fully confirmed in our experimental data.

Other high-ranked genes present on the Starter Array are CCNE1, CCNH (class C), and CHEK1 (class D). CCNE1, another cyclin, is the gene with the highest absolute intensity value in the 24 array [Sutherland and Musgrove, 2004]. It is also expressed after 48 and 72 h, but shows a much lower intensity. A STRING-based bioinformatics prediction suggests it is linked directly with CDC2 (Fig. 1). Indeed, its expression reaches the maximum level in correspondence to one of the process regulated by CDC2, the G1/S transition after 24 h. CCNH, also, is highly expressed 24 h after the stimulation. In fact, the protein encoded by this gene is known to phosphorylate CDC2, thus contributing to the G1/S switching [Karan et al., 2002]. The last considered gene, CHEK1, is an essential kinase required to preserve genome stability. Very recent findings [Syljuasen et al., 2005] proposed that CHEK1 is required during normal S phase to avoid aberrantly increased initiation of DNA replication, thereby protecting against DNA breakage. Our experiments show that CHEK1 expression can be identified 24 and 72 h after the stimulation with PHA, in correspondence to DNA synthesis and mitosis. This can strongly confirm the importance of this gene on DNA repair and preservation mechanisms. Then, we calculated a final map of interactions among these eight high-ranking genes in cell cycle of human T lymphocytes, which is shown in Figure 2, representing also their neighboring genes. The other neighboring genes present in the map are not displayed on the Starter Array. Interestingly, one of them is CDKN1B, which we identified to be a "leader gene" in the control of human T lymphocytes cell cycle [Chang et al., 2004; Sivozhelezov et al., 2006].

CONCLUSIONS

The Starter Array displays only 161 genes: therefore data can be easily analyzed. The use of more complex arrays, displaying a huge amount of genes (up to 10,000) often leads to a difficult and sometimes misleading analysis, due to the complexity of data. Human Starter array allows a simpler analysis. Anyway, genes to be displayed must be chosen with a particular care. Presently we have in progress the construction of DNA chips based on genes identified by bioinformatics, namely the "leaders" of one particular process and "orphan" needing a more detailed attention [Sivozhelezov et al., 2006]. In this way, it becomes possible to create ad hoc arrays, which can guarantee the best results in analyzing a particular cellular system. The here reported "time series" experimentation on human T lymphocytes stimulated with PHA confirm this hypothesis, but is limited by the fact that many interesting genes involved in the human T lymphocytes cell cycle, including three leader genes, are not displayed on the array.

In conclusion, the data on gene expression collected 24, 48, and 72 h after the mitogen stimulus when compared with our bioinformatics prediction [Sivozhelezov et al., 2006] confirmed the theoretical prediction on leader genes. In particular, 24 h after the stimulation, resting T lymphocytes have started cell cycle (MYC), progressed along G1 phase (CDK4) and are preparing to replicate DNA (CDC2); after 72 h, T lymphocytes are about to enter mitosis (CDC2). Moreover, we got a deeper picture of gene expression considering five other genes, whose importance in human T lymphocytes cell cycle was theoretically proven, even if they were not included in the leader gene class. Our

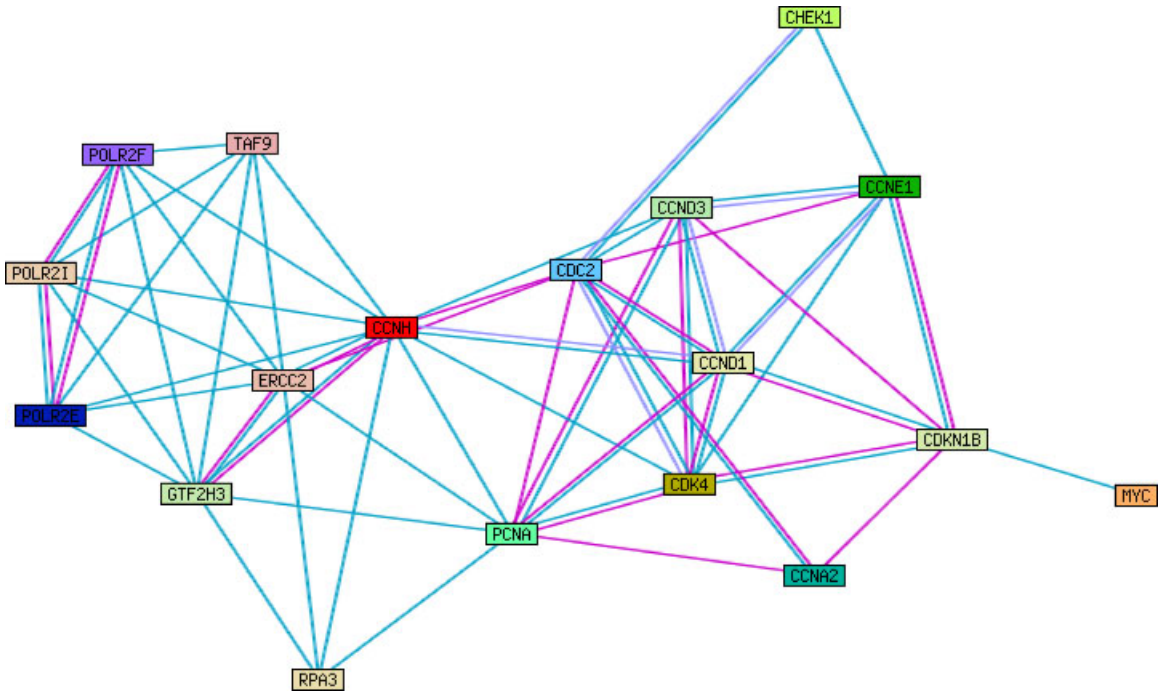

Fig. 2. Final map of interactions among eight high-ranking genes in cell cycle of human T lymphocytes and their neighboring, according to STRING [von Mering et al., 2005]. The lines which connect single genes represent physical interaction between proteins, confirmed by various experimental methods, or the involvement in the same metabolic pathway.

experimental data show a perfect agreement with theoretical ones, therefore further validating the leader gene approach, and also point out the importance of the interaction between PCNA, CDC2, and CCNA2 in controlling cell cycle and of CHEK1 in regulating DNA repair and preservation. At the same time the need of ad hoc array was once again confirmed.

The application of the leader gene approach, starting from the identification of involved genes and their subsequent ranking according to the number of interactions, should be extended to other cellular processes, which are known to a lesser extent if compared with human T lymphocytes cell cycle. Leader genes are defined as the genes with the highest number of interactions among those involved in a particular cellular process. By identifying leader genes of a given process, it becomes possible to design targeted microarray, whose analysis would allow to describe complex biomolecular pathways thorough the activity of a few, but highly important genes, which represent the real center of interactions maps. In this way, an easiest and more rationale approach to molecular genomics can shed new lights on complex cellular mechanisms.

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